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AWARD NUMBER DAMD17-97-1-7228

TITLE: Growth Receptors and Integrins in Breast Cancer

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REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (1 Sep 97 - 31 Aug 98)

4. TITLE AND SUBTITLE

Growth Receptors and Integrins in Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7228

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research And Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The central hypothesis of this IDEA Award is that epidermal growth factor (EGF) receptors in breast cancer transduce intracellular signals that lead to activation of the lipid kinase phosphoinositide 3-OH kinase (PI 3-K) and rapid increases in the functional activity of integrin adhesion receptors, thereby regulating breast cancer cell motility and invasiveness. During this first year of support ,we have demonstrated the overall validity of this hypothesis. EGF and betacellulin, ligands that activate the EGFR, and heregulin-β, a ligand that activates erbB3 and erbB4, induce dose- and time-dependent adhesion of MDA-MB-435 cells to type IV collagen via β1 integrins. These same growth factors also upregulate β1 integrin-mediated migration on collagen and laminin. Inhibition of PI 3-K activity blocks both adhesion and migration induced by these growth factors. Antibody blocking studies further suggest differential recruitment of erbB2 to the erbB3 receptor rather than to the EGFR upon growth factor binding to MDA-MB-435 cells. In other breast carcinoma cell lines, there are intriguing differences in the requirement for PI 3-K in EGF versus heregulin-β-mediated adhesion pathways. Thus, EGFR family members regulate β1 integrin function on breast cancer cells via PI 3-K activation.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
49

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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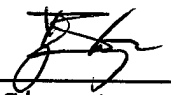

9-27-98

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5. INTRODUCTION

This IDEA Award addresses the critical roles of growth factor receptors and integrin receptors in the regulation of breast cancer cell adhesion, motility and invasion. Many previous studies have examined integrins or growth factors and their receptors as separate entities with respect to the regulation of breast cancer cell growth and metastasis (1-5). Some of these reports documented the alteration of integrin (6) or growth factor receptor (4,5) expression levels on various tumor cells, further emphasizing the potential role of these molecules in tumorigenicity. However, few consistent models have emerged to clarify how these patterns contribute to cellular dysregulation in tumor cells. This may be due in large part to the diverse nature of breast cancer cells and the variation in receptor expression seen across different cell lines. Clearly, a more thorough understanding of the intracellular signals generated by integrins and growth factor receptors in a given cell is needed. We wished to elucidate the pathways that join growth factor receptors and integrins, specifically those that link growth factor signaling to the rapid upregulation of integrin function on breast carcinoma cells.

The β1 integrin family of adhesion receptors plays a critical role in many aspects of cellular adhesion and motility (7-11). Although alterations in levels of integrin expression have been implicated in tumorigenesis, expression, per se, may not necessarily translate into a commensurate functional outcome (12-14). Rapid and transient upregulation of integrin function in response to stimulation of several cell surface receptors has been observed in many cells, particularly circulating leukocytes (12,13,15,16). This regulated activation of integrin function, in the absence of altered cell surface integrin expression, allows for fine-tuned control of cellular adhesive and motile functions, and is exemplified by the regulation of β1 integrins on T cells, as previously characterized by our laboratory and others (12,13,15,17-19). In addition to its structural role, β1 integrins are now appreciated as signaling molecules, with the capacity to receive as well as initiate cascades of signaling events not unlike those of growth factor receptors (9,10). Studies by our laboratory and others have clearly demonstrated the critical role of the lipid kinase phosphoinositide 3-OH kinase (PI 3-K) in the adhesion and signaling events mediated by β1 integrins (19-27).

PI 3-K is a functional heterodimer, consisting of a regulatory p85 subunit and a catalytic subunit of 110 kilodaltons that phosphorylates the D-3 position on the inositol ring of phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate (28,29). Cell surface receptors in a host of cell types and with varying functions interact directly or indirectly with PI 3-K and stimulate its enzymatic activity, thereby generating lipid byproducts that are now believed to participate directly, both in a positive and negative fashion, in the signaling circuitry of cells (30-32). Analysis of PI 3-K function has positioned this enzyme in pathways critical to mitogenesis, cell survival and apoptosis, adhesion, motility, and cytoskeletal reorganization (28,29).

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases activates PI 3-K (33) and plays an important role in the regulation of breast cancer (5). The majority of experimental work on the EGFR family has focused on receptor biochemistry and growth factor-initiated mitogenic signals. However, it is now appreciated that the signaling pathways leading to mitogenesis are likely quite distinct from those of other cellular events such as motility or invasion (34,35). This receptor family is particularly important with regard to the study of breast cancer. First, the EGFR is a member of a complex multisubunit receptor family composed of the EGFR (erbB1), erbB2, erbB3, and erbB4. Members of this receptor family are

overexpressed in a host of human tumors (5), including those of the breast and ovary. Indeed, a variety of clinical studies examined the therapeutic potential of specifically targeting these receptors on tumor cells (36-38). Second, families of growth factors have been described that bind to and initiate signals from distinct EGFR family receptors, potentially generating unique signaling events (39-41). The implication of the ligand-induced heterodimeric signaling of these receptor tyrosine kinases has generated intense investigative interest in recent years, although the potential for differential signaling in cellular adhesion and motility has thus far been unappreciated.

5.A. PURPOSE. The qualitative, rather than quantitative, nature of integrin regulation, specifically in response to growth factor stimulation, has not been fully addressed. The rapid and transient upregulation of integrin function in circulating leukocytes (11) provides a compelling parallel with the metastatic processes undertaken by aggressive tumor cells. In addition, examples of regulation of integrin activation by growth factors such as the c-kit receptor (22,42,43) and platelet derived growth factor receptor (PDGFR) (44) have recently emerged. Additional studies have described the rapid increase in adhesion of a highly metastatic mammary carcinoma cell line to fibronectin (FN) in response to EGF treatment (45). While the studies with PDGFR and c-kit have implicated PI 3-K as a critical signaling component between these growth factor receptors and integrins, the role of PI 3-K has not been investigated with regard to EGFR-stimulated regulation of integrins.

Although several studies have described the activation of PI 3-K in response to EGF treatment (33,40), recent data has suggested that PI 3-K does not directly interact under most circumstances with the EGFR but rather is recruited through other signaling molecules such as $p120^{cbl}$ (cbl) (46,47) or through a dimerization partner such as erbB3 (48,49). Although erbB3 can recruit a variety of signaling molecules through domains in its carboxy-terminal tail, it cannot act as a fully-functional receptor tyrosine kinase in the absence of a dimerization partner due to an enzymatically-impaired kinase domain (50). One critical dimerization partner for both EGFR and erbB3 is erbB2, which is a prognostic indicator in breast cancer. Many breast and ovarian carcinomas overexpress erbB2 at dramatic levels and this overexpression often reflects a poor prognosis in terms of patient survival (51-53). Molecularly, the presence or absence of erbB2 expression correlates with the strength of growth factor signaling (54-57). ErbB4 is the most recently identified member of the EGFR family and is expressed in a more restricted fashion in adult tissues than EGFR, erbB2, and erbB3 (58). However, erbB4 overexpression has been documented in selected tumor types (58), and its regulated expression and function are now beginning to be elucidated (35,59-63).

The role of PI 3-K in EGFR signaling and in the regulation of integrin function in the immune system suggests a potential synergy between EGFR signaling and integrin function in breast cancer. Elucidation of this interaction between EGFR family members and integrins may help to clarify the mechanisms of abnormal cellular processes involved in tumorigenicity and metastasis. Therefore, it has been our aim to dissect the contributions of members of the EGFR family of receptor tyrosine kinases to the regulation of β1 integrin function in breast cancer cells, and to examine the role of PI 3-K in these pathways.

5.B. SCOPE OF RESEARCH. The research aims identified in our original proposal involved two main avenues of experimental effort. Specifically, we hypothesized that: 1) exposure of breast carcinoma cells to EGF would result in generation of an intracellular signaling cascade that would cause a rapid functional upregulation of β1 integrin adhesion receptors; 2)

EGF-dependent activation of integrin function would be dependent on EGFR-mediated activation of the lipid kinase PI 3-K; and 3) inhibition of EGF-mediated activation of PI 3-K would block cancer cell motility and invasiveness induced by EGF-dependent activation of β1 functional activity.

Our proposed analysis of EGFR signaling to the β1 integrins included: 1) characterization of EGFR family and β1 integrin subunit expression on various breast cancer cell lines, 2) examination of EGF-induced PI 3-K activation in breast cancer cells, 3) analysis of changes in β1 integrin affinity states upon EGF stimulation of cells, 4) examination of β1 avidity changes by observing cell spreading and cytoskeletal reorganization in response to EGF, and 5) analysis of EGF-mediated cell motility and invasion. Analysis of the role of PI 3-K in EGF-mediated activation of β1 integrin function was proposed in the following set of approaches: 1) assessment of pharmacological inhibition of PI 3-K and the resulting effect on EGF-activation of integrins and 2) examination of molecularly inhibiting or enhancing PI 3-K function using constitutively active and dominant negative forms of PI 3-K transfected into breast carcinoma cells.

6. BODY

6.A. EXPERIMENTAL METHODS.

Cell lines. The MDA-MB-435S, MDA-MB-231, and MDA-MB-361 cells were maintained in Leibovitz's L-15 medium (Gibco) supplemented with 10% fetal calf serum (FCS, Atlanta Biologicals). SKBR3 cells were grown in McCoy's 5a medium (Celox Laboratories) containing 15% FCS. A431 cells were grown in Dulbecco's Modified Essential Media (DMEM; Gibco) with 10% FCS. MCF7 cells were maintained in 90% Eagle's MEM (Mediatech), 1X non-essential amino acids (Mediatech), 1 mM Na Pyruvate (Sigma), 10 μg/ml insulin (Gibco) and 10% FCS. T47D cells and the 528 hybridoma, expressing the anti-EGFR monoclonal antibody, were maintained in RPMI 1640 medium (Mediatech) containing 10% FCS. Culture supernatant was harvested from overconfluent cultures of 528 cells and was titered for detection of the EGFR. All cell lines were obtained from ATCC and all cell culture media contained additives of 2 mM L-glutamine, and 50 U/ml penicillin/streptomycin (Mediatech).

Flow cytometry. Single-color flow cytometric analysis (FACS) was performed on cells in suspension after removal from tissue culture flasks with EDTA or trypsin. 5×10^5 cells were typically analyzed with antibodies incubated as 1 μg purified antibody, 5 μl ascites antibody, or 25 μl antibody in culture supernatant/1×10^6 cells. Antibodies in the form of ascites or culture supernatant were routinely titered for appropriate detection of cell surface receptors. Antibodies for flow cytometric analysis included: the anti-EGFR monoclonal antibody 528 (ATCC), the anti-erbB2 monoclonal Ab-5 (Calbiochem), the anti-erbB3 monoclonal antibody Ab-4 and the anti-erbB4 monoclonal antibody Ab-1 (Lab Vision, Inc.), the β1 integrin-specific monoclonal antibody TS2/16 (ATCC), the β2-integrin-specfic monoclonal antibody TS1/18 (ATCC), the α1-integrin-specific monoclonal TS2/7 (ATCC), the α2-integrin-specific monoclonal antibody P1E6 (Gibco), the α3-integrin-specific monoclonal antibody P1B5 (Gibco), the α4-integrin-specific monoclonal antibody NIH49d-1 (kind gift of Dr. S. Shaw), the α5-integrin-specific monoclonal antibody P1D6 (Gibco), the α6-integrin-specific monoclonal antibody GoH-3 (ICN/Cappell), and FITC-conjugated goat anti-mouse IgG or goat anti-rat IgG (Southern Biotechnology, Inc.). Cells in FACS buffer (Hanks buffered saline solution (HBSS), containing 1% bovine calf serum (BCS; Hyclone Laboratories, Inc.)) were incubated with appropriate antibodies for 30 minutes on ice, washed 3 times in FACS buffer, and incubated for an additional 30 minutes with

appropriately diluted FITC-conjugated secondary antibodies. After 2 washes in ice-cold FACS buffer, data was acquired on a Becton Dickinson FACScan or FACScalibur and analyzed using Cellquest software.

DNA constructs and transfections. The GFP-wild type p85 and GFP-Δp85 constructs have been previously described (23). Transfections were carried out by electroporation in 4 mm gap cuvettes (Invitrogen). 5 X 10^6 cells in 300 μl Opti-MEM (GIBCO) were incubated with 25 μg appropriate DNA and electroporated using 250V, 960 μF settings on a BioRad gene pulser with capacitance extension. After allowing the cells to recover for 20 minutes at room temperature, cells were transferred to tissue culture flasks containing 20% FCS, 80% L-15 media and were allowed to recover for 24-48 hours prior to use in adhesion or migration assays. Typical transient expression of DNA constructs ranged from 15-35% of recovered cells.

Adhesion assays. Standard adhesion assays were performed using cells labeled with Calcein AM (Molecular Probes) as previously described (27). Extracellular matrix ligands were human type IV collagen (Sigma), human merosin or laminin (Gibco), and human FN. For transient expression of GFP-fusion proteins, adhesion was quantitated following collection of adherent cells and analysis by flow cytometry essentially as described (23,64). Growth factor stimulation was performed with EGF (Gibco), betacellulin, HRGα, or HRGβ (all from R&D Systems). For receptor blocking studies, cells were incubated in the presence of the anti-β1 integrin antibody P5D2 (kind gift of T. LeBien), the anti-erbB2 Ab-16, the anti-erbB3 Ab-5, or the anti-erbB4 Ab-3 (all from Lab Vision, Inc.) at 1 μg antibody/1 X 10^6 cells or as indicated in figure legends. Tyrphostin AG1478 (Calbiochem) was used for inhibition of the EGFR. Pharmacological inhibition of PI 3-K was performed with wortmannin (Sigma) or LY294002 (Alexis Corp.). Inhibition of MEK was performed using the inhibitor PD98059 (Parke-Davis).

Migration assays. Cell lines were allowed to grow to suconfluency (about 75-85%) prior to harvest for migration studies. Subconfluent cell cultures were placed in serum-free media for 12-24 hours and harvested by releasing from flasks with 1 mM EDTA. After cells were washed free of EDTA in serum-free RPMI 1640 media, they were quantitated and assessed for viability using trypan blue. Cells at a density of 400,000 cells/ml in assay media (RPMI, 20 mM HEPES, 0.1% BSA) were added in 57 μl to the upper well of a 48-well chemotaxis chamber (Neuro Probe, Inc.), containing assay media or appropriate growth factor. Polycarbonate filters (8 μm; Osmonics) were precoated with mouse EHS-collagen or EHS-laminin (Gibco) at 20 μg/ml in PBS overnight at 4°C and allowed to air dry before placing in chambers. Cells were allowed to migrate in the presence or absence of stimulators for 4-6 hours at 37°C before disassembly of the chambers, fixing and staining of the migrated cells. Nonmigrated cells were removed from the upper surface of the filters after placing on a microscope slide, and cell migration was quantitated by counting and taking the sum of migrated cells in 4 separate fields of at least 3 individual wells. For inhibition studies, cells were preincubated for 15 minutes on ice with inhibitor or appropriate control prior to addition to chemotaxis chambers.

For transient transfection/migration assays cells were transfected as described above. Cells were serum-starved for 12 hours prior to harvesting for migration assays. Transwell chambers (6-well size, 8 μm filters, Costar) or 8 μm polycarbonate membrane filters for Boyden chemotaxis chambers were coated overnight at 4°C in solutions of mouse EHS-laminin or EHS-collagen at 20 μg/ml in PBS. Growth factors diluted in assay media were added to the lower wells of chemotaxis chambers or transwells, and coated filters were placed on top. Cells were then added to upper wells at approximately 1 X 10^6 cells/well in 1.5 ml assay media. The same

dilution of cells was used for addition to quadruplicate wells in 24-well plates (100 µl/well) and to the upper wells of Boyden chambers, and migration was allowed to proceed overnight in both setups. Boyden chemotaxis chambers were disassembled and analyzed the following morning as described above. Transwell migration chambers were disassembled, and migrated cells were removed from the lower surface of each well with 1:1 trypsin:EDTA. Dislodged cells were added to FACS tubes containing ice-cold 10% FACS buffer (HBSS, 10% bovine calf serum), spun and resuspended in 200 µl 10% FACS buffer. Cells plated in 24-well plates were also harvested and placed into FACS tubes for approximation of cells added/well and for determining the % efficiency of transfectants. FACS tubes containing representative starting cell populations or migrated cell populations were analyzed by flow cytometry with additions of 25,000 reference beads/tube (9.7 µm; Interfacial Dynamics) to determine cell numbers present in each tube. Quantitation was done essentially as described (23,64)

Immunoprecipitation. Cells that had been serum-starved for 12-24 hours were harvested from tissue culture flasks using 1 mM EDTA. Cells were washed in serum-free RPMI 1640 to remove EDTA and were quantitated by trypan exclusion. Equal aliquots of cells were added to eppendorf tubes and stimulated in the presence or absence of growth factors for the indicated periods of time at 37°C. Following stimulation, cells were lysed directly in 1 ml lysis buffer (1% Triton X-100, 1% deoxycholic acid, 158 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.2, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM sodium orthovanadate) on ice for 20 minutes. Supernatants were clarified by centrifugation for 20 minutes at 4°C and post-nuclear supernatants were immunoprecipitated with the antiphosphotyrosine antibody PY20 (10 µl 1:10 ascites, provided by Dr. M. Kamps, University of California, San Diego, CA) overnight at 4°C. Protein-A sepharose (Zymed; 50 µl/tube) was added the following morning for an additional 1 hour incubation, and immunocomplexes were washed twice in lysis buffer containing protease inhibitors. Protein-A-sepharose bound proteins were boiled for 4 minutes in the presence of 2X SDS-sample buffer (125 mM Tris, pH 6.8, 4% sodium dodecyl sulfacte (SDS), 2 mM EDTA, 20% glycerol, 10% β-mercaptoethanol (2-ME), 0.6% bromphenol blue) and were separated on 10% polyacrylamide gels by SDS-polyacrylamide gel electrophoresis (PAGE).

Western blotting. Cell lysates or immunoprecipitates were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.0075% SDS) for 2 hours at 400 mA. Membranes were incubated in blocking buffer (5% Carnation milk, PBS) for 1 hour at room temperature or overnight at 4°C. Blots were rinsed in PBS prior to addition of primary antibodies diluted in blocking buffer (4G10; UBI, anti-EGFR sc-03; Santa Cruz, anti-p85; UBI, anti-erbB3 Ab-7; Lab Vision) for 1 hour at room temperature. Blots were rinsed 3 times in PBS, 0.1% Tween-20 for 10 minutes each before addition of secondary antibodies diluted in blocking buffer (goat anti-mouse IgG-horse radish peroxidase (GAM-IgG-HRP, Gibco) or donkey anti-rabbit-IgG-HRP (DAR-IgG-HRP; Amersham)) for 1 hour at room temperature. Blots were rinsed 3 times in PBS, 0.1% TWEEN-20 and bands were visualized using enhanced chemiluminescence (Pierce Chemical). For reprobing membranes, stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 0.1M 2-ME) was used at 50°C for 30 minutes followed by blocking membranes in 5% milk, PBS, and reprobing with appropriate antibodies.

6.B. RESULTS AND DISCUSSION

Adhesion of breast carcinoma cell lines to β1 integrin ligands can be regulated by stimulation of EGFR family members. In order to test our hypothesis that EGF stimulation might

upregulate β1 integrin function in breast cancer cells, we examined a variety of breast carcinoma cell lines, both metastatic and non-metastatic in nature, for their ability to respond to EGF stimulation by upregulating adhesion to β1 integrin extracellular matrix (ECM) ligands. Several cell lines were examined, including the highly metastatic lines MDA-MB-435 (65), MDA-MB-361, and SKBR3, and the less metastatic cell lines MDA-MB-231 (65,66), T47D, MCF7 (66,67), and A431. The cell lines responded in a heterogeneous fashion with regard to adhesion to matrix ligands such as FN, Type IV collagen (COLL), and laminin (LAM) or merosin (MER). For example, EGF treatment increased adhesion of MDA-MB-435 cells to FN, COLL, MER, and LAM (Figure 1A). However, in line with previous observations (45), low doses of EGF had little effect on A431 cell adhesion and a negative effect at higher doses of EGF for adhesion of A431 cells to FN (Figure 1B) or COLL (Figure 1C). While the A431 cell line dramatically overexpresses the EGFR, other cell lines with high levels of EGFR, such as SKBR3 and MDA-MB-231, were not inhibited in adhesion by stimulation with EGF in our assays, further underscoring the complexity of these regulatory pathways in a given defined cell system. Indeed, the SKBR3 cells showed significantly increased adhesion to FN and COLL and a smaller increase on MER in response to EGF (Figure 1D), while MDA-MB-231 cells routinely showed little stimulated adhesion to FN or COLL (Figure 1E). However, the MDA-MB-435, T47D (Figure 1F) and SKBR3 cells routinely showed increased adhesion to these ECM components in response to EGF stimulation. However, all of the cell lines showed increased adhesion in response to the activating β1 integrin-specific mAb TS2/16 (68,69), which directly binds to the β1 integrin subunit and induces a conformational change that increases adhesion (Figure. 1). This suggests that the heterogeneous response of these cell lines to EGF is due primarily to differences in EGFR signaling, rather than inherent differences in β1 integrin function. Because of the inherent complexity involved in studying multiple cell lines, and due to the observation of strong β1 dependent adhesion on collagen for the MDA-MB-435 cells, the majority of our initial studies focused on the regulatory pathways between the EGFR family and β1 integrins in this cell line.

Adhesion of MDA-MB-435 cells to type IV collagen was specifically induced by exposure to the growth factor EGF, although to less extent than by directly activating the β1 integrin with TS2/16 (68,69). β1 integrins were the major adhesion receptors responsible for this event as indicated by the nearly complete inhibition of both unstimulated and stimulated (TS2/16 or EGF) adhesion to collagen by these cells (Figure 2A). Adhesion to FN and MER was typically blocked less effectively with inhibitory β1 integrin-specific antibodies, leaving open the possibility of contributions by other adhesion receptors. The EGF-mediated adhesion to collagen was dose dependent, with maximal stimulation of adhesion peaking at 10 ng/ml EGF with a plateau of maximal adhesion at higher concentrations of growth factor (Figure 2B). Based on these early findings, we typically stimulated cells with 100 ng/ml EGF in cellular adhesion assays. The EGF-stimulated adhesion to collagen was also induced in a rapid fashion, with maximal increases over unstimulated adhesion found by 10-20 minutes of stimulation at 37°C (Figure 2C).

MDA-MB-435 cells express moderate levels of the EGFR, erbB2, and erbB3, with no detectable erbB4 protein by flow cytometry or blotting methods (not shown). Since erbB2 is still considered an "orphan" receptor in that no growth factor has yet been found by which erbB2 is directly bound and activated, the presence of both EGFR and erbB3 on the surface of these cells led us to investigate additional growth factors that bind to and activate either the EGFR or erbB3. Betacellulin stimulation of the EGFR also increased MDA-MB-435 cell adhesion to collagen in a dose-dependent fashion, with the maximal stimulation of adhesion paralleling that found with

EGF stimulation in the same assay (Figure 3A). Examination of heregulin-alpha (HRGα), a growth factor that binds only to erbB3 or erbB4, showed no effects on adhesion of this cell line at any of the concentrations of growth factor tested (Figure 3B). In contrast, the more recently described heregulin isoform, heregulin-beta (HRGβ), showed a striking increase in adhesion of these cells to collagen with increasing levels of growth factor (Figure 3C). The stimulatory effect of HRGβ was slightly less than direct activation of β1 through the TS2/16 antibody, and was routinely higher than EGF stimulation. These data suggested that stimulation through either the EGFR with ligands such as EGF or betacellulin, or stimulation of the erbB3 receptor with HRGβ, could significantly stimulate β1 integrin-dependent adhesion of the MDA-MB-435 cell line to type IV collagen. Importantly, this upregulation occurred in the absence of significant changes in either integrin subunit or EGFR family receptor numbers on the cell surface as assessed by flow cytometry (data nor shown).

Stimulation of EGFR family members increases migration of MDA-MB-435 breast carcinoma cells. As the MDA-MB-435 cells are highly metastatic in nude mouse models (65), we also examined cellular migration and motility *in vitro*. Previous studies described migration and adhesion of unstimulated MDA-MB-435 cells on laminin and collagen (3), and our FACS analyses shows strong expression of β1 integrins capable of binding collagen and laminin (α2, α3, α6; data not shown), consistent with this earlier study (3) Additionally, both collagen and laminin represent critical physiological components for cellular metastatic motility (70-72). Thus, we examined the migration of unstimulated and stimulated MDA-MB-435 cells on both collagen and laminin. As found in our adhesion experiments, betacellulin, EGF, and HRGβ all increased the migration of 435 cells toward laminin (Figure 4A) in a dose-dependent fashion, while parallel assays showed similar results on collagen (data not shown). While the adhesion experiments showed increasing growth factor-stimulated adhesion with a plateau response (Figure 2A and Figure 3A), betacellulin and EGF both demonstrated bell-shaped curves for stimulated migration, with maximal responses at 0.1-1 ng/ml and 1-10 ng/ml for betacellulin and EGF, respectively. Migration in response to HRGβ showed similar dose-effects as seen for adhesion with strong induction of migration, reaching a maximal response by 100-250 ng/ml growth factor. While the stimulated adhesion by HRGβ was consistently higher than that mediated by EGF, both events were β1 integrin-dependent as illustrated by the ability of an inhibitory β1 integrin-specific antibody to completely abrogate both unstimulated and EGF- or HRGβ-stimulated migration towards collagen or laminin (Figure 4B). Thus, our adhesion and migration data support our hypothesis that growth factor stimulation of the EGFR couples to β1 integrin-mediated functional events. Further, we have found that erbB3, another receptor in the EGFR family, mediates stimulation of both adhesion and cell migration by the growth factor HRGβ.

Contribution of dimerization partners with the EGFR and erbB3 in EGF and HRGβ regulation of β1 integrins. The observation that two mechanistically distinct growth factors, EGF and HRGβ, were capable of stimulating β1 integrin activity, coupled with the complex heterodimerization potential of the EGFR family of receptors (39,41), led us to investigate the potential contribution of dimerization partners with the EGFR or with erbB3 in mediating the effects of EGF and HRGβ on β1 integrin function. An anti-erbB3 antibody that blocks HRGβ binding (73) specifically abrogated HRGβ-induced adhesion of MDA-MB-435 cells without affecting EGF or TS2/16 stimulated adhesion, even at high concentrations of antibody (Figure 5A and data not shown). Additionally, an anti-erbB2 antibody that blocks the effects of EGF or HRGβ binding to the dimerization partners of erbB2 (74) negated HRGβ-stimulated adhesion without specifically affecting EGF or TS2/16 stimulation conditions (Figure 5A). The

combination of both anti-erbB3 and anti-erbB2 antibodies gave a slightly stronger reduction in adhesion, further supporting the contribution of both erbB2 and erbB3 in HRGβ-induced adhesion. Although we could not detect erbB4 protein expression in our experiments with the MDA-MB-435 cells, it was possible that low, but undetectable levels of erbB4 might be mediating the HRGβ effects that we observed. However, an antibody that blocks HRGβ binding to erbB4 (73) did not inhibit HRGβ-induced adhesion, even when combined with the erbB3 blocking antibody (Figure 5B). Although no effects of the erbB2 or erbB3 blocking antibodies were seen on EGF-mediated adhesion, only EGF-mediated adhesion was abrogated by the highly EGFR-specific inhibitor tyrphostin AG1478 (75)in a dose-dependent fashion (Figure 5C). At a concentration of 50-100 nM inhibitor, the EGF-induced adhesion was reduced to approximately the levels of unstimulated cell adhesion with little effect observed on the ability of HRGβ or TS2/16 to stimulate maximal adhesion.

Because of the strong stimulation of cell migration initiated by HRGβ, we extended our antibody blocking studies to COLL and LAM migration assays to determine the receptor subunits contributing to these signals. Similar to the adhesion assays, anti-erbB3 and anti-erbB2 antibodies blocked HRGβ-stimulated MDA-MB-435 cell migration toward laminin (Figures 6A and 6B) or collagen (data not shown). In addition, incubation of cells with the AG1478 tyrphostin had negligible effects on either unstimulated or HRGβ-stimulated cell migration (Figure 6C). Although we have not yet fully examined the EGF-mediated migration pathways in MDA-MB-435 cells, an interesting picture of preferential partnering with erbB2 is suggested by these studies for EGFR- versus erbB3-mediated signaling of adhesion or migration. While erbB2 is a favored dimerization partner for the EGFR, even over EGFR-EGFR homodimers (54,55), we did not observe any significant inhibition of EGF-stimulated adhesion by the erbB2 antibody. This suggests that for cellular adhesion and migration, erbB2 may have more of a role as a conduit for erbB3-initiated signals, while the EGFR does not require erbB2 for the more moderate effects on adhesion in these cells.

Role of PI 3-K in EGF- and HRGβ-induced upregulation of β1 integrin function. As PI 3-K plays a role in signaling by the EGFR family members, we analyzed the contributions of this enzyme to the adhesion and migration events we had observed in the MDA-MB-435 cells. Stimulation of MDA-MB-435 cells with either EGF (Figure 7A) or HRGβ (Figure 7B) results in rapid recruitment of the p85 subunit of PI 3-K to the phosphotyrosine-containing cellular fraction, consistent with previous reports of growth factor stimulated PI 3-K activation in other cell lines (76). We tested the relevance of PI 3-K activation to adhesion with two pharmacologically distinct PI 3-K inhibitors, wortmannin and LY294002 (77-80). When cellular adhesion assays were performed in the presence of either 100 nM wortmannin (Figure 8A) or 25 μM LY294002 (Figure 8B) we observed a significant, although incomplete, decrease in both EGF- and HRGβ-stimulated MDA-MB-435 cell adhesion to collagen. In contrast, only small reductions in TS2/16-induced or unstimulated adhesion were observed. PI 3-K appears to contribute even more significantly to the process of migration in these cells as both inhibitors markedly reduced HRGβ-mediated migration on laminin (Figure 8 C and D) as well as collagen (data not shown). In these experiments, unstimulated migration was also reduced, but only at the highest doses of the inhibitors. While cells generally migrated in smaller numbers in experiments using EGF as a stimulus, motility induced by EGF on COLL and LAM was also strongly inhibited by wortmannin or LY294002 (Figure 8E).

In order to further support our pharmacological inhibitor data, we employed a transient transfection assay that allowed us to assess the adhesion and migration of untransfected as well

as transfected cells by flow cytometric analysis of adherent or migrated cell populations (23,64). Control vector expressing green fluorescent protein (GFP) alone or constructs expressing GFP-tagged wild type p85 or a dominant negative p85 subunit (Δp85) were transiently transfected into MDA-MB-435 cells followed by analysis in a modified cell adhesion assay. As shown in Figure 9, increasing levels of GFP alone (C2) had little effect on the adhesion of MDA-MB-435 cells under any stimulation condition, while expression of either wtp85 or Δp85 subunits of PI 3-K decreased EGF or HRGβ-mediated adhesion by approximately 50% without affecting TS2/16 or unstimulated adhesion significantly.

The effects of molecularly inhibiting PI 3-K function on HRGβ-stimulated cell migration of MDA-MB-435 cells was also investigated. Comparison of control transfected or Δp85-transfected cell migration in standard Boyden chamber conditions revealed some inhibitory effect of Δp85 expression on HRGβ-mediated cell migration, while no striking inhibition of unstimulated migration was apparent (Figure 10A). However, analysis of the specific GFP-positive, Δp85-positive cells in comparison to GFP-negative cells showed a striking inhibition of both HRGβ-stimulated and unstimulated cell migration with increasing expression of the Δp85 construct (Figure 10B), in keeping with our results using wortmannin and LY294002. Thus, both EGF- and HRGβ-stimulated pathways require functional PI 3-K for optimal β1 integrin-mediated adhesion and migration of MDA-MB-435 cells.

Role of EGFR family members in regulating β1 integrin function on other breast carcinoma cell lines. While the focus of our work in the first year was on MDA-MB-435 cells as a model system for growth factor regulation of integrin-mediated adhesion and migration, we made several compelling observations during the course of analyzing other cell lines in our studies. Our adhesion assays showed consistent EGF-induced increases in adhesion of the T47D cell line to collagen as well as FN (Figure 1E), both of which were completely blocked by inhibitory β1 integrin-specific antibodies (Figure 12 A, B). However, two observations suggest that these cells are mechanistically distinct from the MDA-MB-435 cells. First, while adhesion to FN can be stimulated by EGF, betacellulin, or HRGβ, only adhesion stimulated by the EGF family ligands (EGF and betacellulin) was sensitive to the PI 3-K inhibitors wortmannin and LY294002 (Figure 11). In contrast to our results with MDA-MB-435 cells (Figures 8, 9 and 10), HRGβ-induced adhesion of T47D cells was insensitive to both of these inhibitors (Figure 11). Second, while the EGFR family blocking antibodies were quite effective in elucidating the dimerization partners mediating erbB3 versus EGFR signaling in MDA-MB-435 cells, these same antibodies were completely ineffective at abrogating the EGF- or HRGβ-stimulated adhesion of T47D cells to FN or collagen (Figure 12), even at 10X the typical dose of 1 μg antibody/1 X 10^6 cells (Figure 12C and D). T47D cells express EGFR, erbB2, and erbB3 at levels similar to MDA-MB-435 cells, but with the additional expression of erbB4. However, erbB4-blocking antibodies were also not effective alone or in combination in reducing HRGβ or EGF-stimulated adhesion of these cells (Figure 12A and B).

While both MDA-MB-435 and T47D cells express modest levels of the growth factor receptors we have examined, SKBR3 cells express relatively high levels of EGFR and erbB3. To our surprise, we observed no inhibition of HRGβ or EGF-stimulated adhesion of SKBR3 cells to collagen or merosin by the anti-erbB3 or anti-erbB4 antibodies (Figure 13A, B). However, the anti-erbB2 blocking antibody appeared to effectively abrogate EGF-stimulated adhesion of SKBR3 cells to collagen or merosin without affecting HRGβ-stimulated adhesion (Figure 13 C and D). Although we often noted some increase of unstimulated adhesion in the presence of the erbB2 blocking antibody (compare Figure 12 C and D and Figure 13 C and D),

EGF-stimulated adhesion was routinely reduced to the levels of unstimulated adhesion in the presence of the blocking antibody in at least three separate experiments. Thus, preliminary analyses of growth factor stimulated upregulation of β1 integrin function across several cell lines has hinted at the complexity in regulation both by signaling pathways utilizing PI 3-K, and in the receptor-receptor specificity of interactions that transmit signals from bound EGF or HRGβ to the cellular network governing adhesion and migration.

6.C. RECOMMENDATIONS RELATED TO ORIGINAL PROPOSAL

In the first aim of this IDEA Award, we proposed analyzing several parameters related to breast cancer cell adhesion, motility, and invasion. We have accomplished many of our initial objectives in this aim in the first year and generated a body of compelling data that has helped to redefine key points remaining to be addressed within the context of this proposal. First, we proposed screening a variety of cell lines for integrin and growth factor receptor expression, to assess the activation of PI 3-K in these cell lines, and to examine the nature of their adhesion to various ECM ligands. Using FACS and blotting analyses, we characterized the integrin and EGFR family profiles of several breast cancer cell lines. Preliminary adhesion data revealed a subset of breast cancer cell lines that were informative from the standpoint of upregulated adhesion in response to growth factor stimulation. We further characterized those lines, including MDA-MB-435, T47D and SKBR3 cells. We observed activation of PI 3-K upon EGF or HRGβ stimulation of MDA-MB-435 cells, as indicated by the recruitment of PI 3-K to the phosphotyrosine-containing cellular fraction. At this time, we have not examined each cell line for PI 3-K activation in response to EGF or HRGβ due to the desire to focus our efforts on a more narrowly-defined system. However, these studies remain in our consideration as we continue to examine the differences between the cell lines of interest, particularly the differences in sensitivity to wortmannin and LY294002 we have noted for the T47D cells.

We also examined adhesion events to several ECM ligands, including FN, type IV collagen, merosin, and laminin. While we could demonstrate growth factor-induced adhesion on all of these ECM components to differing degrees, we focused our efforts on β1 integrin-dependent adhesion of MDA-MB-435 cells to type IV collagen, in the hopes of more clearly understanding the repertoire of molecules interacting in the context of a single cell type. We have also made significant progress with our studies on cellular migration, using the MDA-MB-435 cells as our current model system. These studies have shown clear stimulation of migration on both collagen and laminin upon activation with EGF family ligands (EGF or betacellulin). In addition, we have demonstrated the dramatic upregulation of breast cancer cell adhesion and migration in response to treatment with the erbB3 and erbB4 ligand, HRGβ, and have described the preferential recruitment of erbB2 to erbB3 heterodimers over the EGFR when signals are initiated from HRGβ or EGF, respectively.

The second aim of this proposal is to examine the role of PI 3-K in EGF-regulation of β1 integrin function. Using the two approaches that were originally suggested, we have demonstrated a clear role for PI 3-K in not only the EGF upregulation of integrin-mediated adhesion and migration but also those stimulated by HRGβ. Instead of an inducible expression system as originally suggested for expression of dominant negative forms of PI 3-K, we employed transient expression of PI 3-K mutants combined with modified adhesion (23,64) and migration assays and analysis of specific cell populations by flow cytometry as a means of supporting the pharmacological data we had generated. We have not yet formally demonstrated inhibition of EGF-stimulated MDA-MB-435 cell migration by dominant-negative p85 using this

method. However, given the strength of effect on HRGβ-mediated migration, and the consistent results we observed for both EGF and HRGβ-stimulated adhesion in the presence of transiently expressed wtp85 or Δp85, we fully anticipate that EGF-stimulated migration will be strongly inhibited by this same approach.

While evidence exists that PI 3-K is involved in unstimulated invasion by MDA-MB-435 cells (81), our work has now demonstrated EGFR family members also play an important role in regulating adhesion and migration mediated by β1 integrins. Our results are in contrast to another study that analyzed breast epithelial cell migration in response to EGF (82). In this report, transfectants overexpressing the EGFR showed a dramatic increase in EGF-stimulated cell migration in comparison to parental ZR75-1 cells. Incubation with wortmannin and LY294002 enhanced EGF-mediated migration of EGFR-overexpressing cells, while the MEK1 inhibitor PD98059 decreased EGF-stimulated adhesion. However, no effect of either inhibitor was observed on the parental cells, reported to express approximately 20,000 EGFR on the cell surface as compared to 1,200,000 EGFR on the transfected cells. Our results suggest that PI 3-K plays a positive role in both stimulated and unstimulated adhesion and migration of MDA-MB-435 cells, and we have noted little effect of inhibitors affecting the MAPK pathway (data not shown), as reported by others (81). Altogether, these observations underscore the need to further clarify the role of the PI 3-K pathway and to understand its contributions to the signals generated by a given repertoire of EGFR family receptors expressed on a given cell type.

In addition, we have made preliminary observations in a second cell line, T47D, of differential regulation of EGF and HRGβ-driven pathways, exemplified by differing sensitivities to the PI 3-K inhibitors wortmannin and LY294002. While T47D cells express similar levels of EGFR, erbB2, and erbB3 to those found in MDA-MB-435 cells, they express the additional HRGβ receptor, erbB4, which is not expressed on MDA-MB-435 cells. Recent reports have described unique proteolytic processing of erbB4 (61,62,83) as well as at least one alternate transcript, found in both normal breast tissue and in several breast tumors, that lacks a putative p85 binding site (63). The relevance of this observation with regard to our studies is currently unclear but warrants further investigation. Divergent signals generated from ligand binding to erbB3 compared to the EGFR appears likely, given the unique nature of the erbB3 cytoplasmic tail, which recruits a unique panel of phosphoproteins compared to that of the EGFR. For example, erbB3 does not bind to PLCγ or GAP (84), and preferentially interacts with Grb7 rather than Grb2 (85). ErbB3 also contains at least 6 sites in its cytoplasmic tail that are considered to be optimal for binding the SH2 domain of p85. In addition, varying reports have described mitogenic, growth-inhibitory, or differentiative effects of HRG stimulation depending on the growth factor isotype, the concentration used and the cell line studied(54,86-88). Thus, the current understanding of erbB3 and erbB4 signaling upon HRG binding reveals the need for a better understanding of their contributions to a variety of cellular processes, including cell adhesion and migration.

Our initial proposal outlined additional experimental avenues that remain for future endeavors in the coming year. These include the examination of growth factor stimulation on affinity and avidity changes of β1 integrins as well as invasion studies to further our observations of cell migration. In addition, we believe that further study is warranted to pursue the observation we have made regarding differential sensitivity of EGF or HRGβ-mediated pathways in at least one cell line to PI 3-K inhibition. Given the current complexity of the EGFR family of receptors and their roles in the various cellular processes contributing to tumor cell formation and metastatic growth, it will be important to more clearly delineate the dimerization

events undertaken by members of the EGFR family following the binding of specific ligands in a way that is informative for the signals generated by those ligands to upregulate β1 integrins. Finally, several additional molecules of interest could be investigated for their contribution to EGF or HRGβ-mediated pathways. These include cbl, R-Ras, and rac1 and cdc42.

While we have not yet focused our efforts on the cbl molecule, it remains of interest in both PI 3-K-dependent and -independent pathways leading to upregulated adhesion and migration. Indeed, recent work from our laboratory in the study of human lymphocytes has suggested a role for cbl in β1 integrin-mediated adhesion events (21). In addition, cbl has recently been implicated in the regulation of growth factor receptor signaling as demonstrated by its role in the ubiquitin-dependent downregulation of PDGF-receptors following growth factor stimulation (89). These studies may be particularly relevant to our system given the observation that cbl is primarily recruited to the EGFR and not to other members of the EGFR family (90)and that the EGFR is significantly downregulated in response to ligand binding while erbB2, erbB3, and erbB4 are not regulated in the same fashion (91,92). Finally, studies in *C. elegans* (93) and Drosophila (94) suggest that cbl is a negative regulator of the EGFR, and recent functional studies in mammalian cells have implicated the JAK/STAT pathway rather than the raf pathway in mediating cbl's regulatory effect on the EGFR (95). This is consistent with our preliminary data showing little effect of a MEK inhibitor on EGF or HRGβ-mediated adhesion or migration (data not shown). We plan to pursue studies over the next year to test the specific role of cbl in coupling the EGFR to PI 3-K-dependent regulation of adhesion and migration in breast cancer cell lines.

Additional preliminary data from our laboratory suggest a regulatory role for the R-ras molecule on the adhesion of lymphocyte cell lines (unpublished observations). R-ras has transforming properties in certain cell types (96,97) and couples to some of the same signaling pathways as H-ras. However, it has also shown unique regulation in, for example, its inability to couple to the Sos regulatory molecule or to activate Raf (98-101). While little is yet known about the contributions of R-ras to cellular adhesion and migration pathways, at least one report has suggested a regulatory role for R-ras on integrins (102). A recent report has also implicated R-ras as an important participant in cellular motility, demonstrating increased cell scattering of epithelial cells in the presence of constitutively active R-ras (103). Importantly, PI 3-K mediates the primary pathway involved in this response, utilizing downstream effectors distinct from rac and Akt. This is also consistent with studies demonstrating that R-ras can activate PI 3-K but not MAP kinase (98). We have wild-type and mutant forms of R-ras available as GFP fusion proteins that we will test to see if they alter growth factor-mediated regulation of β1 integrin function.

Finally, the contributions of Rho-family proteins such as rac1 and cdc42 to cellular cytoskeletal changes such as membrane ruffling and lamellipodia formation have been appreciated for some time (104), but their placement in signaling pathways between growth factor receptors and integrin activation is relatively unexplored. These signaling molecules, and various dominant negative or constitutively active forms, are currently available for our use, most being based in a vector to produce GFP-fusion proteins, lending themselves well to our transient expression-adhesion and migration assays. Thus, we will begin exploring the potential role these molecules may have in EGF or HRGβ signaled events to β1 integrins in breast carcinoma cells and to determine their contribution to the PI 3-K pathways initiated by growth factor binding to the EGFR family of receptor tyrosine kinases.

7. CONCLUSIONS

Our data have clearly shown the ability of growth factors that bind to and activate the EGFR family of receptor tyrosine kinases to upregulate cellular adhesion and migration processes via activation of β1 integrins. We have shown that that EGF and betacellulin, ligands that activate the EGFR, and HRGβ, a member of the neuregulin family of ligands that bind to and activate erbB3 and erbB4, induce dose- and time-dependent adhesion of MDA-MB-435 cells to type IV collagen. This adhesion is mediated by β1 integrins, and functional PI 3-K is required. These same growth factors also upregulate β1 integrin-mediated migration on collagen and laminin, again utilizing PI 3-K-dependent pathways. Studies with blocking antibodies have further suggested a differential recruitment of erbB2 to the erbB3 receptor rather than to the EGFR upon growth factor binding. In other breast carcinoma cell lines, there are differences in the requirement for PI 3-K in EGF versus HRGβ-mediated adhesion pathways. Further, the contributions by various EGFR family receptor subunits to EGF or HRGβ signaling across different cell lines may be unique. The molecular basis for these differences is under investigation. Thus, our work over the past year has confirmed the validity of our initial hypothesis regarding the regulation of β1 integrins by EGFR family members in breast cancer, and the role of PI 3-K in this signaling cascade. In addition, our work has generated many new questions for experimental pursuit in the coming year.

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9. APPENDICES

9.A. FIGURE LEGENDS

Figure 1. EGF modulates breast carcinoma cell adhesion to ECM ligands. MDA-MB-435 (A), A431 (B and C), SKBR3 (D), MDA-MB-231 (E), or T47D cells (F) were analyzed for their ability to respond to EGF treatment in cell adhesion assays performed on fibronectin (FN), type IV collagen (COLL), merosin (MER), or mouse EHS-laminin (EHS-LAM). Cells that had been serum starved for 12-24 hours were harvested by release from tissue culture flasks in 1 mM EDTA. Suspended cells were than washed in serum-free media to remove excess EDTA prior to quantitation and labeling with Calcein AM as previously described (27). Adhesion assays were performed in 96-well plates that were precoated overnight at 4°C with extracellular matrix (ECM) ligands as indicated. ECM proteins were coated at 1 μg/well unless otherwise noted. Cells were than added to wells containing assay media alone or stimulators: the activating β1 integrin-specific monoclonal antibody, TS2/16, or EGF at 100 ng/ml final concentration or at increasing amounts as indicated (B and C). Cells were allowed to settle briefly in wells before analyzing preadherent fluorescence on a fluorescence plate reader. Cells were then stimulated at 37°C for 10 minutes before removing nonadherent cells by hand washing with a syringe/manifold system. Cells plated on BSA alone as a control for nonspecific adhesion generally showed less than 10% adhesion (data not shown). Comparison of adhesion to human type IV collagen or mouse EHS-collagen, or human laminin and mouse EHS-laminin did not show significant differences (data not shown).

Figure 2. EGF-stimulated adhesion of MDA-MB-435 cells to type IV collagen is mediated by β1 integrins and shows dose and time dependence for growth factor stimulation. MDA-MD-435 cells were serum-starved before harvesting for adhesion assays essentially as described for Figure 1. Cells were preincubated at 4°C with control mouse IgG or with the inhibitory β1 integrin-specific antibody P5D2 at 1 μg/1 X 10^6 cells for 10 minutes prior to addition to plates coated with 1 μg/well COLL, 1 μg/well FN or 0.3 μg/well MER (A). For analysis of dose response to growth factor, increasing amounts of EGF ranging from 1 pg/ml to 500 ng/ml final concentration were added to COLL-coated wells prior to addition of cells and stimulation at 37°C. Binding to BSA-coated wells was performed as a control (B). The time-course of adhesion to COLL was determined in the presence of 1 μg/well TS2/16 or 100 ng/ml EGF in comparison to unstimulated adhesion (PBS).

Figure 3. MDA-MB-435 cell adhesion is stimulated by additional growth factors that bind to and activate members of the EGF receptor family. Adhesion to COLL was determined in the presence of increasing amounts of the EGF-like growth factor betacellulin (A), or growth factors that bind specifically to erbB3 and erbB4, heregulin-α (B) and heregulin-β (C). Unstimulated (PBS), EGF- or TS2/16-stimulated adhesion were analyzed for comparison.

Figure 4. Betacelluin, EGF, and heregulin-β induce β1 integrin-dependent MDA-MB-435 cell migration on laminin. Cells were grown to approximately 75% confluency and placed in serum-free media for 16 hours before harvesting as for adhesion assays. Polycarbonate filters (8 μm) were coated overnight at 4°C in PBS containing mouse EHS-laminin or EHS-collagen at 20 μg/ml. 48-well chemotaxis chambers were assembled with assay media alone or containing increasing amounts of growth factors in the lower chambers, as indicated (A). LAM-coated filters were placed over the lower wells and approximately 23,000 MDA-MB-435 cells were placed in the upper wells and allowed to migrate at 37°C for 4-6 hours. Migrated cells in each

well were quantitated on fixed and stained filters. The sum of 4 microscopic fields was taken for each well, and 3 wells were averaged for each stimulation condition. Antibody blocking studies were carried out by preincubating cells with control IgG or the inhibitory β1 integrin-specific antibody P5D2 at 1 μg/1 X 10^6 cells on ice for 10 minutes before addition to the upper wells of chambers containing EGF or HRGβ, and containing filters coated with EHS-LAM or EHS-COLL (B).

Figure 5. Heregulin-β mediated adhesion of MDA-MB-435 cells to collagen requires heterodimerization with erbB2. Adhesion assays were performed as previously described. To examine contributions by erbB2, erbB3 or erbB4, control IgG or specific monoclonal blocking antibodies were incubated with cells prior to addition to plates containing TS2/16, EGF, or HRGβ (A and B). EGF signaling was examined by stimulating cells with PBS, TS2/16, EGF, or HRGβ in the presence of increasing amounts of the EGFR-specific tyrphostin AG1478 or DMSO control.

Figure 6. Heregulin-β mediated migration of MDA-MB-435 cells on laminin requires heterodimerization with erbB2. Migration assays were carried out as described for Figure 4. To examine contributions by erbB3 (A) or erbB2 (B), control IgG or specific blocking antibodies were incubated with cells prior to addition to chemotaxis chambers containing control media or HRGβ. The contribution by the EGFR to HRGβ mediated migration was examined by stimulating cells with control media or HRGβ in the presence 100 nM tyrphostin AG1478 or DMSO control.

Figure 7. EGF and HRGβ stimulation of MDA-MB-435 cells induces recruitment of the p85 subunit of PI 3-K to the phosphotyrosine cellular fraction. Cells were serum-starved for 24 hours and harvested as previously described. 7.5 X 10^6 cells/sample were incubated in the absence or presence of 100 ng/ml EGF (A) or 100 ng/ml HRGβ (B) for the indicated periods of time at 37°C. Cells were then lysed, and phosphotyrosine-containing proteins were immunoprecipitated with the anti-phosphotyrosine monoclonal antibody PY20. Washed immunocomplexes were separated by SDS-PAGE and transferred to PVDF membranes. Western blotting was performed on membranes with anti-phosphotyrosine monoclonal antibody 4G10 to detect phosphotyrosine-containing proteins (upper panels). Blots were stripped and reprobed for EGFR or erbB3 (A and B, respectively, data not shown), and for p85 (lower panels).

Figure 8. PI 3-K inhibitors block EGF or HRGβ-mediated adhesion and migration of MDA-MB-435 cells. Adhesion (A and B) or migration (C, D and E) assays were carried out in the presence of wortmannin (A, C and E) or LY294002 (B, D, and E). Adhesion assays were performed in the presence of 100 nM wortmannin or 25 μM LY294002. Migration assays on laminin for HRGβ stimulation (C and D) were performed in the presence of increasing amounts of either inhibitor as indicated. Similar effects were observed when dose response analysis was carried out on collagen (data not shown). EGF-stimulated migration on COLL or LAM (E) was performed in the presence of control DMSO, 100 nM wortmannin, or 25 μM LY294002.

Figure 9. Overexpression of the wild type or dominant negative p85 subunit inhibits EGF- or HRGβ-mediated increases in MDA-MB-435 adhesion to collagen. Control vector expressing GFP (C2) or constructs expressing a GFP-wild type p85 (wt p85) or GFP-Δp85 (deltap85) fusion protein were transiently transfected into MDA-MB-435 cells as described in Materials and Methods. Transfected cells were allowed to recover for 24 hours and then placed

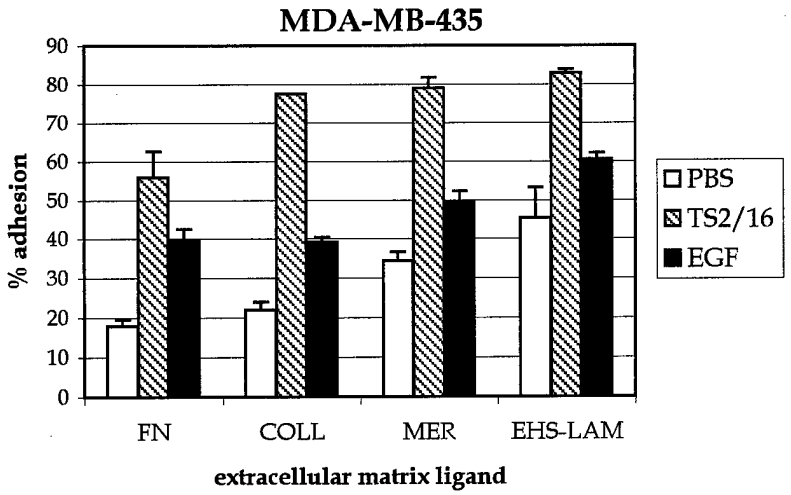
in serum-free media overnight. Cells were harvested as for standard adhesion assays except than no Calcein AM labeling was performed and cells (approximately 300,000 cells/well) were added to 24-well plates coated with 6 μg/well COLL. Adhesion was analyzed in the presence of PBS alone, or containing 1 μg/well TS2/16, 100 ng/ml EGF or 100 ng/ml HRGβ for 10 minutes at 37°C. Nonadherent cells were washed away and adherent cells were collected from wells using a 1:1 trypsin:1 mM EDTA solution. Collected cells were then analyzed by flow cytometry using aliquots of preadherent cell populations to confirm cell numbers added to wells for each transfectant and to determine the % expression of GFP in the starting cell populations. Percent adhesion was determined by gating GFP-negative, GFP-low, -middle-, and -high-positive cells and comparing preadherent and adherent cell numbers from each population. The data shown reflect fold differences in adhesion when compared to the GFP-negative, unstimulated cell subpopulation from each transfectant. Average % adhesion was determined from samples examined in triplicate for each stimulation condition.

Figure 10. Overexpression of the dominant negative p85 subunit inhibits HRGβ-mediated increases in MDA-MB-435 migration on laminin. Control vector expressing GFP (C2) or a construct expressing a GFP-Δp85 (deltap85) fusion protein were transiently transfected into MDA-MB-435 cells. Transfected cells were allowed to recover for 24 hours and then placed in serum-free media for 24 hours. Cells were harvested and quantitated as previously described. Standard chemotaxis analysis was performed as described in Materials and Methods with aliquots of cells at the same concentration as used for migration in transwells (below). Cells were allowed to migrate in the presence or absence of HRGβ on laminin-coated filters overnight, and migrated cells were fixed, stained, and quantitated as described (A). The migration of GFP-positive transfected cells was assessed specifically by allowing cells to migrate overnight in transwell chambers that had been precoated with LAM. Cells were incubated in the presence or absence of HRGβ at 100 ng/ml in the lower wells. After approximately 16 hours, migrated cells were collected from the lower surface of LAM-coated filters with trypsin/EDTA and analyzed by flow cytometry as described for transient adhesion assays (B). Data represent the average of 3 wells/conditions and show the fold difference in migration as compared to the GFP-negative, unstimulated cell migration.

Figure 11. Stimulated adhesion of T47D cells to FN mediated by EGF-family ligands or HRGβ displays differential sensitivity to PI 3-K inhibitors. T47D cells were grown to subconfluency and serum-starved for 12 hours in RPMI 1640. Adhesion assays using Calcein AM labeled cells were carried out in the presence or absence of 10 ng/ml betacellulin (BCELL), 100 ng/ml EGF, or 100 ng/ml HRGβ. Additionally, cells were exposed to either control DMSO, wortmannin (100 nM), or LY294002 (25 μM) for 10 minutes at 37°C prior to removal of nonadherent cells and quantitation of flourescence in the adherent cell populations.

Figure 12. T47D cells adhesion to FN or COLL is increased by stimulation with EGF or HRGβ and is β1 integrin-dependent, but cannot be abrogated by anti-erbB2, anti-erbB3, or anti-erbB4 blocking antibodies. Subconfluent cultures of T47D cells were serum-starved for 24 hours and labeled with Calcein AM following harvesting with 1 mM EDTA as described previously. Adhesion assays were carried out in 96-well plates that had been precoated overnight at 4°C with 1 μg/well FN (A and C) or 1 μg/well COLL (B and D). Blocking antibodies recognizing β1 integrins (P5D2), erbB2, erbB3, or erbB4 were incubated with cells at 1 μg/1 X 10^6 cells or at increasing amounts as indicated. Cells were then stimulated to adhere in the presence or absence of 1 μg/well TS2/16, 100 ng/ml EGF, or 100 ng/ml HRGβ. Adherent cells were quantitated by fluorescence following plate washing as previously described.

Figure 13. SKBR3 breast carcinoma cell adhesion to COLL or MER is increased by EGF or HRGβ stimulation and is β1 integrin-dependent, but only EGFR-activated signals require heterodimerization with erbB2. Subconfluent cultures of SKBR3 cells were serum-starved overnight and harvested in 1 mM EDTA. Cells were quantitated, labeled with calcein AM, and incubated on ice with blocking antibodies recognizing β1 integrins (P5D2), erbB2, erbB3, or erbB4. Antibody-coated cells were than added to 96-well plates coated with 0.3 μg/well collagen (A and C) or 0.3 μg/well merosin (B and D) in the presence or absence of TS2/16, EGF, or HRGβ.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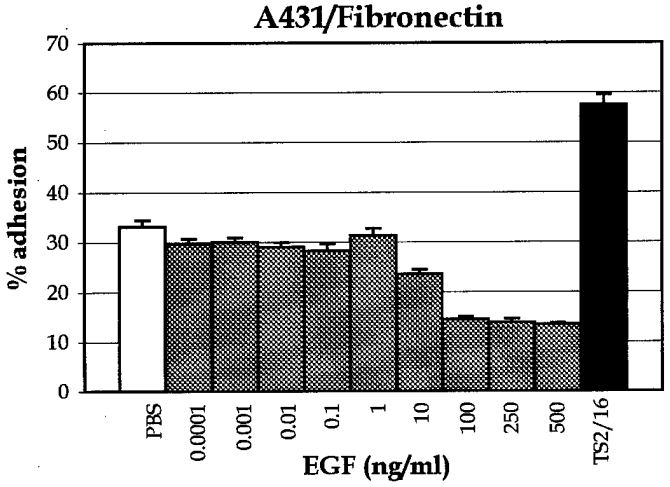
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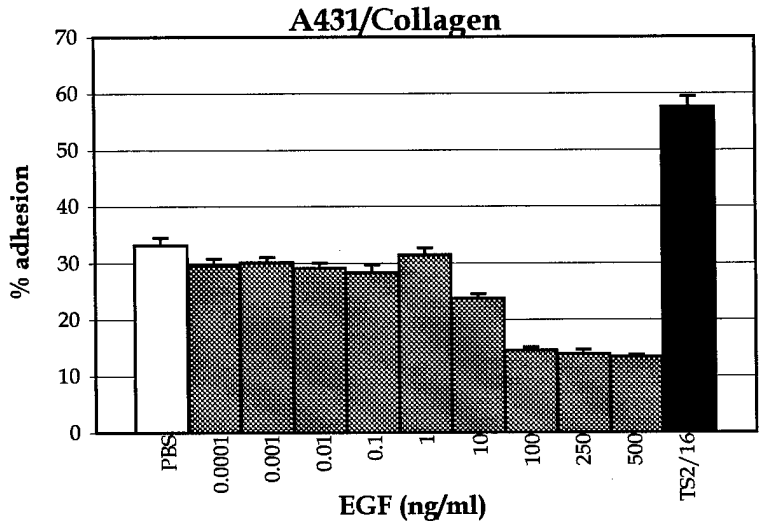
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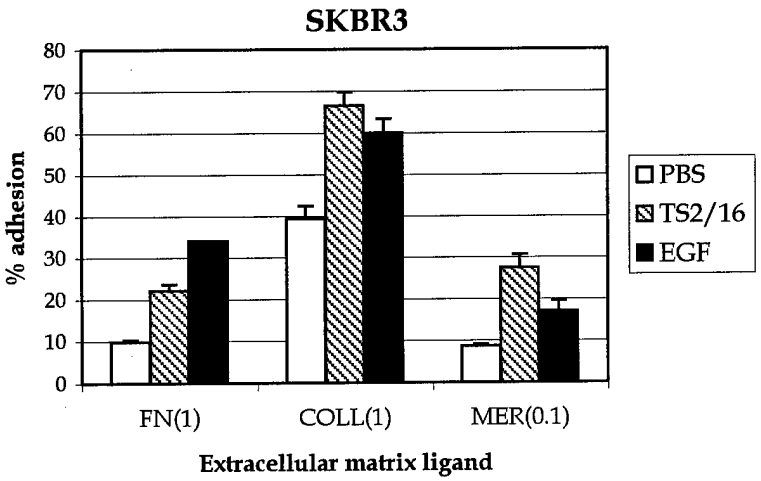
Figure 1



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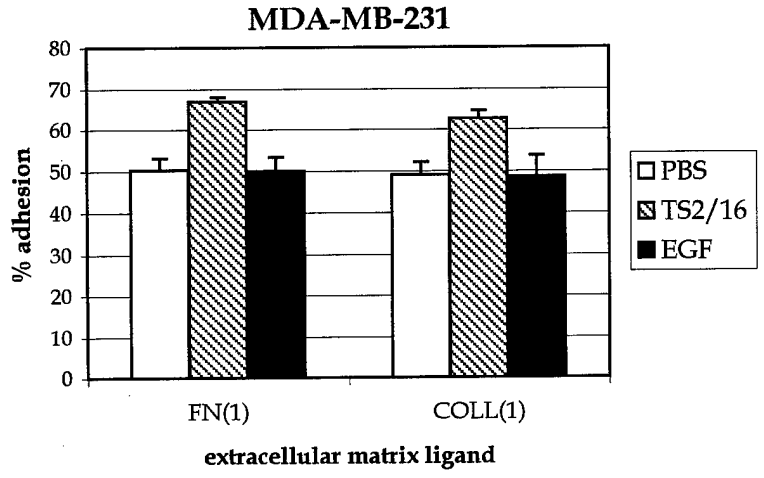

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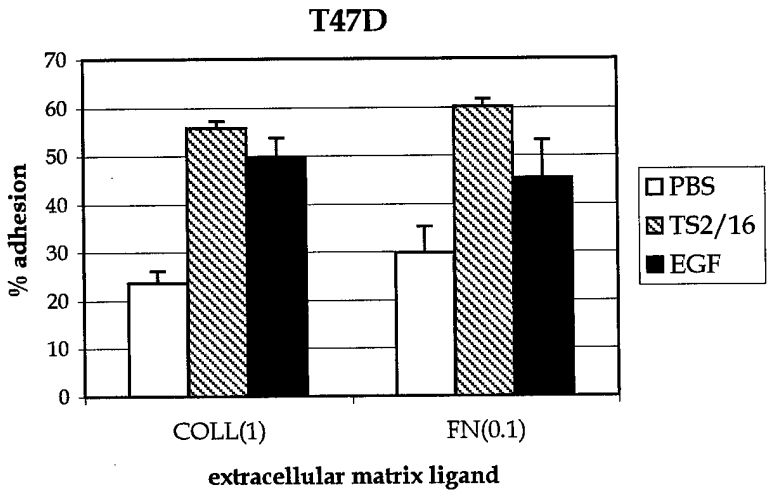

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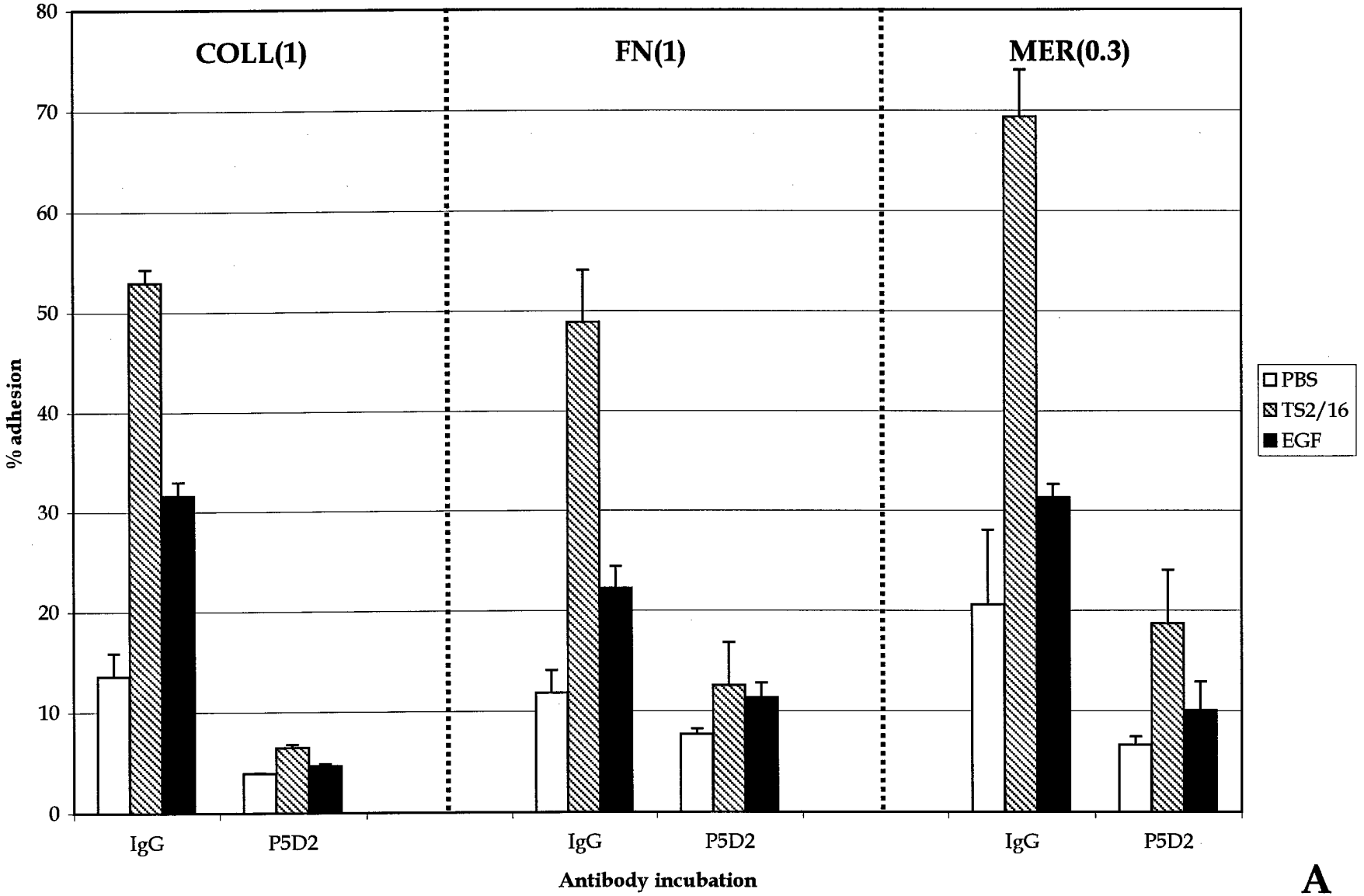
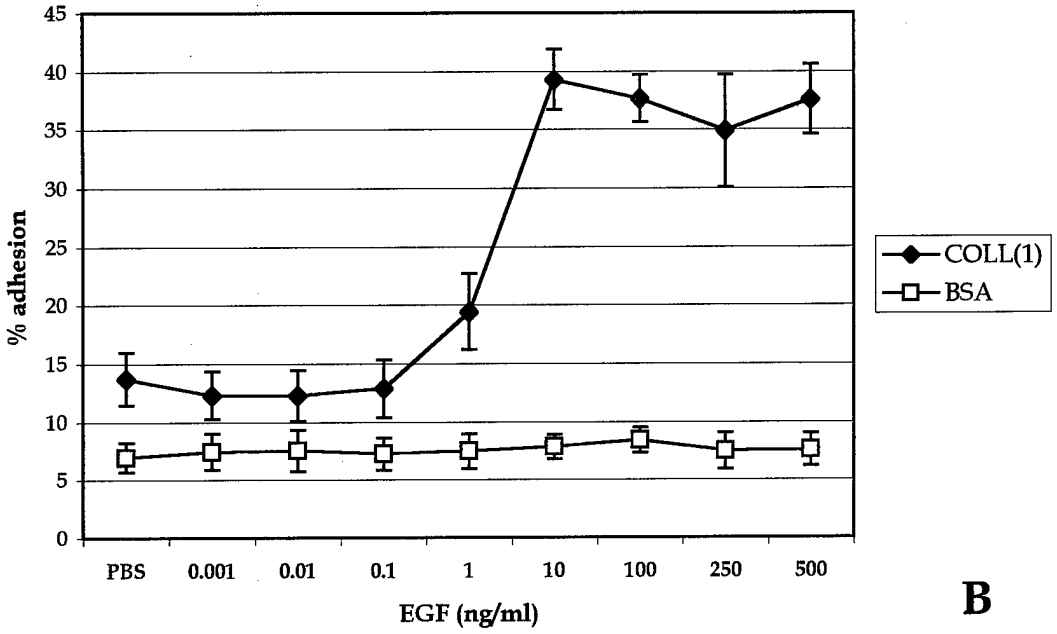
Figure 1 (cont)

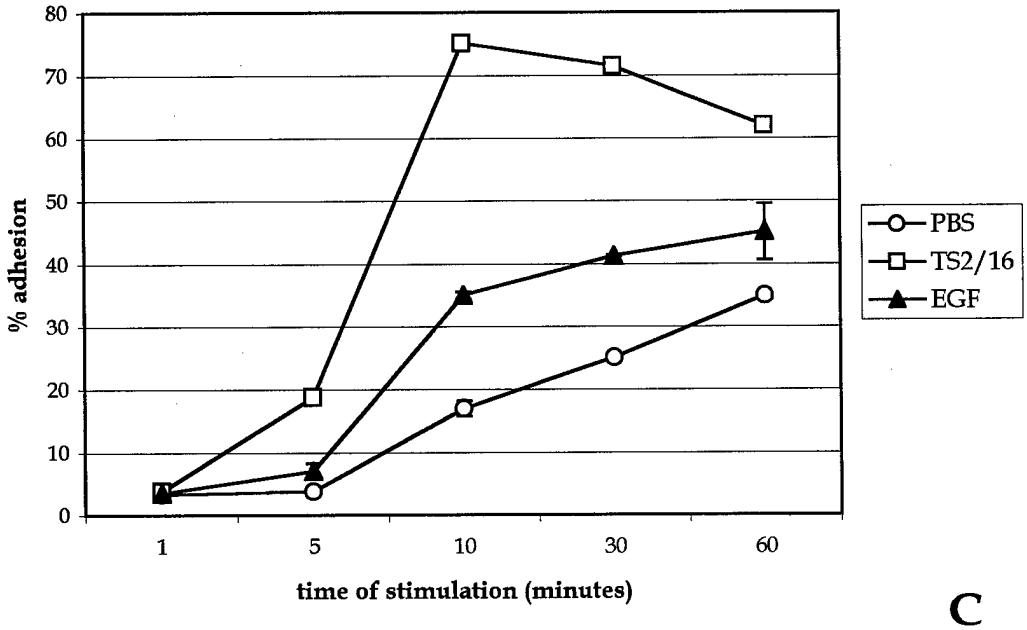

COLL(1)
FN(1)
MER(0.3)
% adhesion
80
70
60
50
40
30
20
10
0
IgG
P5D2
IgG
P5D2
IgG
P5D2
Antibody incubation
PBS
TS2/16
EGF

A

Figure 2

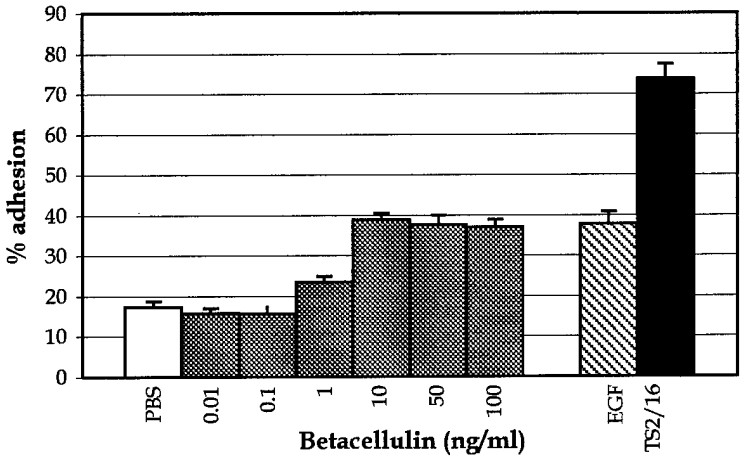


B

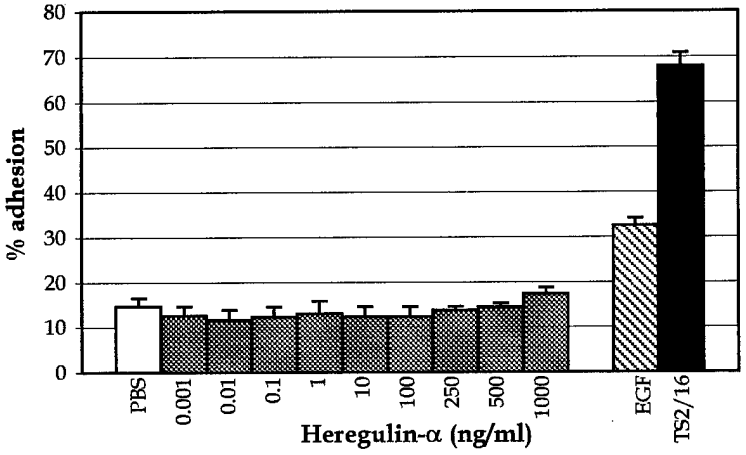


C

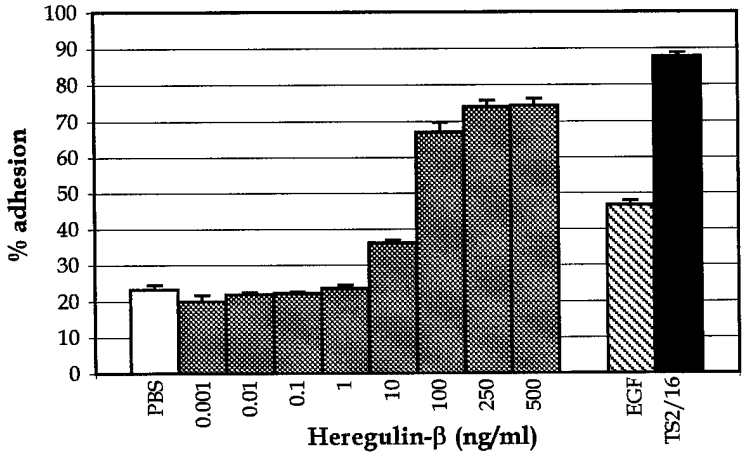
Figure 2 (cont)


90
80
70
60
50
40
30
20
10
0
% adhesion
PBS
0.01
0.1
1
10
50
100
EGF
TS2/16
Betacellulin (ng/ml)

A

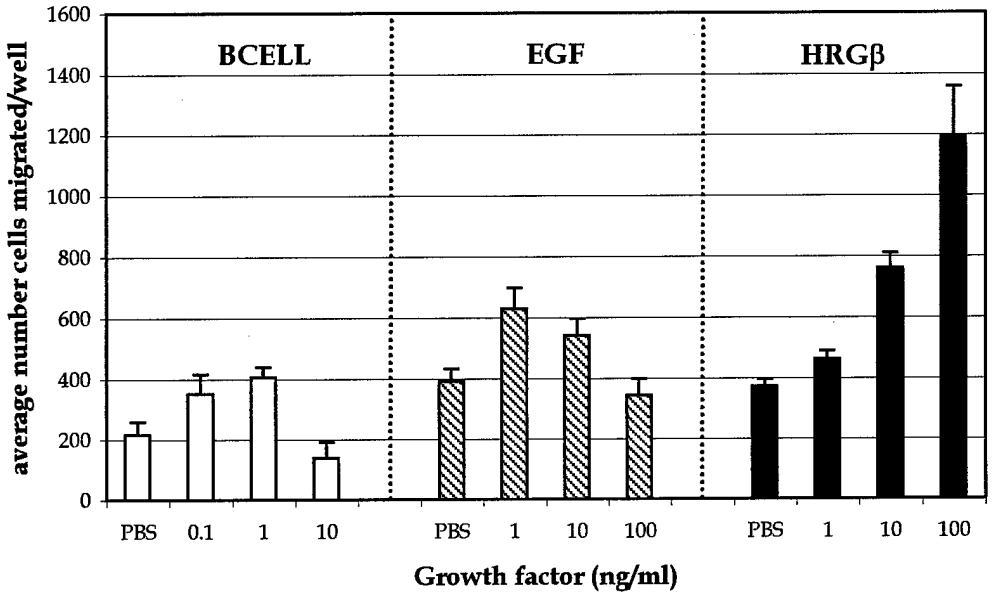

80
70
60
50
40
30
20
10
0
% adhesion
PBS
0.001
0.01
0.1
1
10
100
250
500
1000
EGF
TS2/16
Heregulin-α (ng/ml)

B

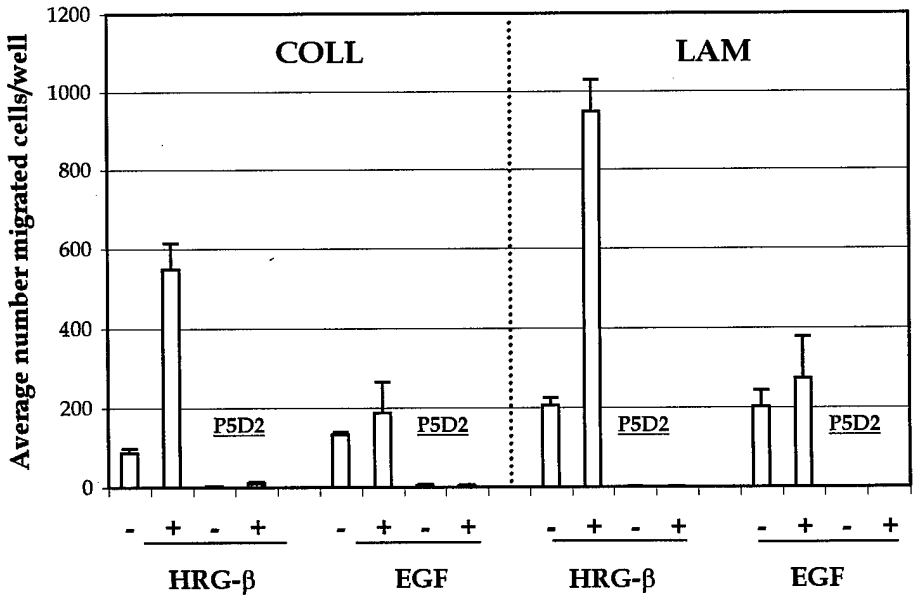

100
90
80
70
60
50
40
30
20
10
0
% adhesion
PBS
0.001
0.01
0.1
1
10
100
250
500
EGF
TS2/16
Heregulin-β (ng/ml)

C

Figure 3

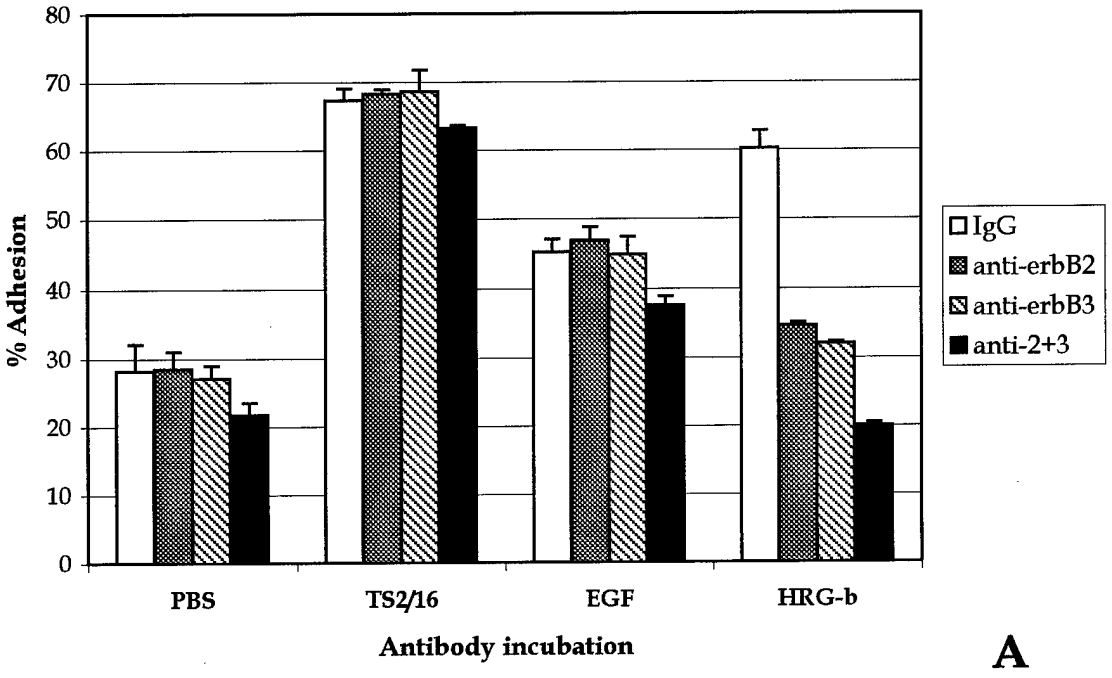

BCELL
EGF
HRGβ
average number cells migrated/well
1600
1400
1200
1000
800
600
400
200
0
PBS
0.1
1
10
PBS
1
10
100
PBS
1
10
100
Growth factor (ng/ml)

A

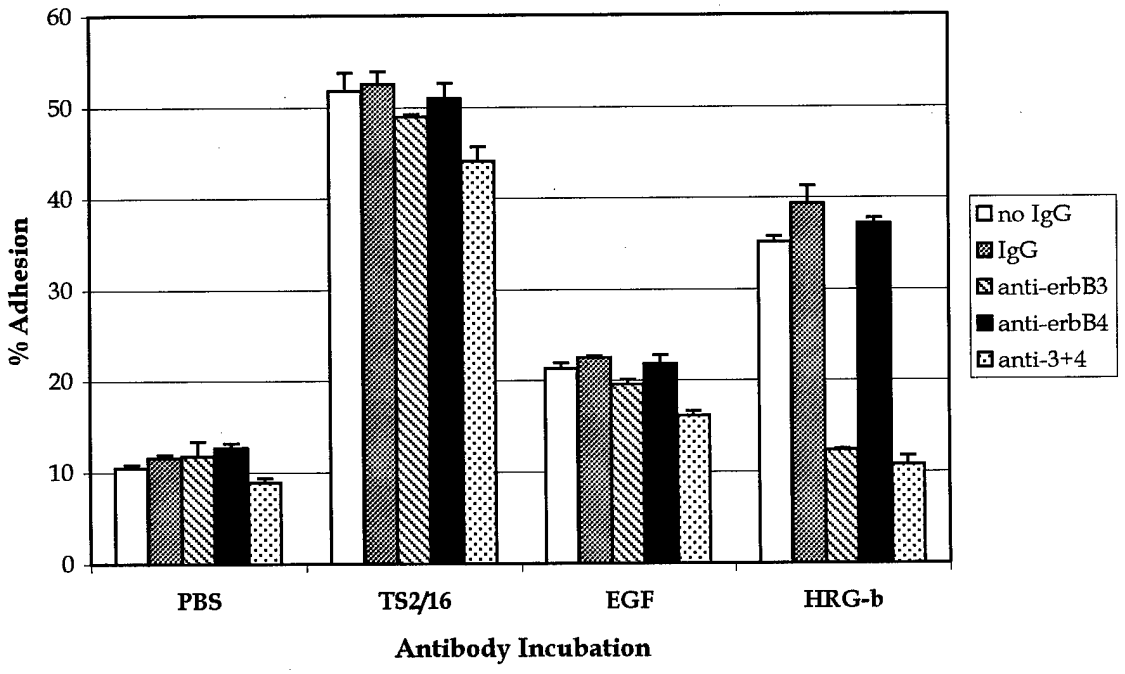

COLL
LAM
Average number migrated cells/well
1200
1000
800
600
400
200
0
P5D2
P5D2
P5D2
P5D2
- + - +
- + - +
- + - +
- + - +
HRG-β
EGF
HRG-β
EGF

B

Figure 4

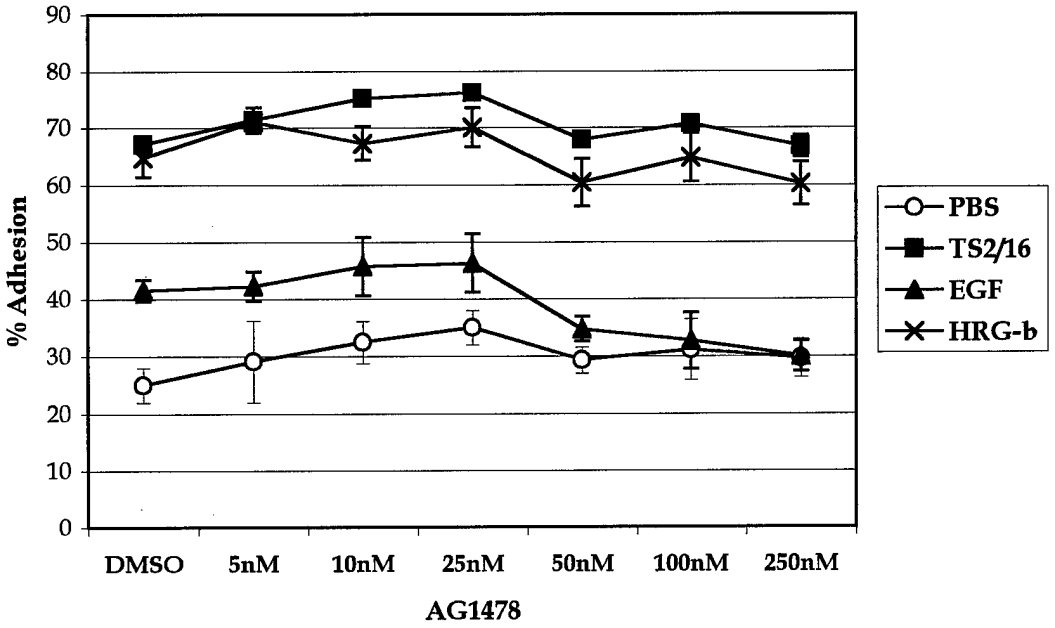

80
70
60
50
40
30
20
10
0
% Adhesion
IgG
anti-erbB2
anti-erbB3
anti-2+3
PBS
TS2/16
EGF
HRG-b
Antibody incubation

A


60
50
40
30
20
10
0
% Adhesion
no IgG
IgG
anti-erbB3
anti-erbB4
anti-3+4
PBS
TS2/16
EGF
HRG-b
Antibody Incubation

B

Figure 5



C

Figure 5 (cont)

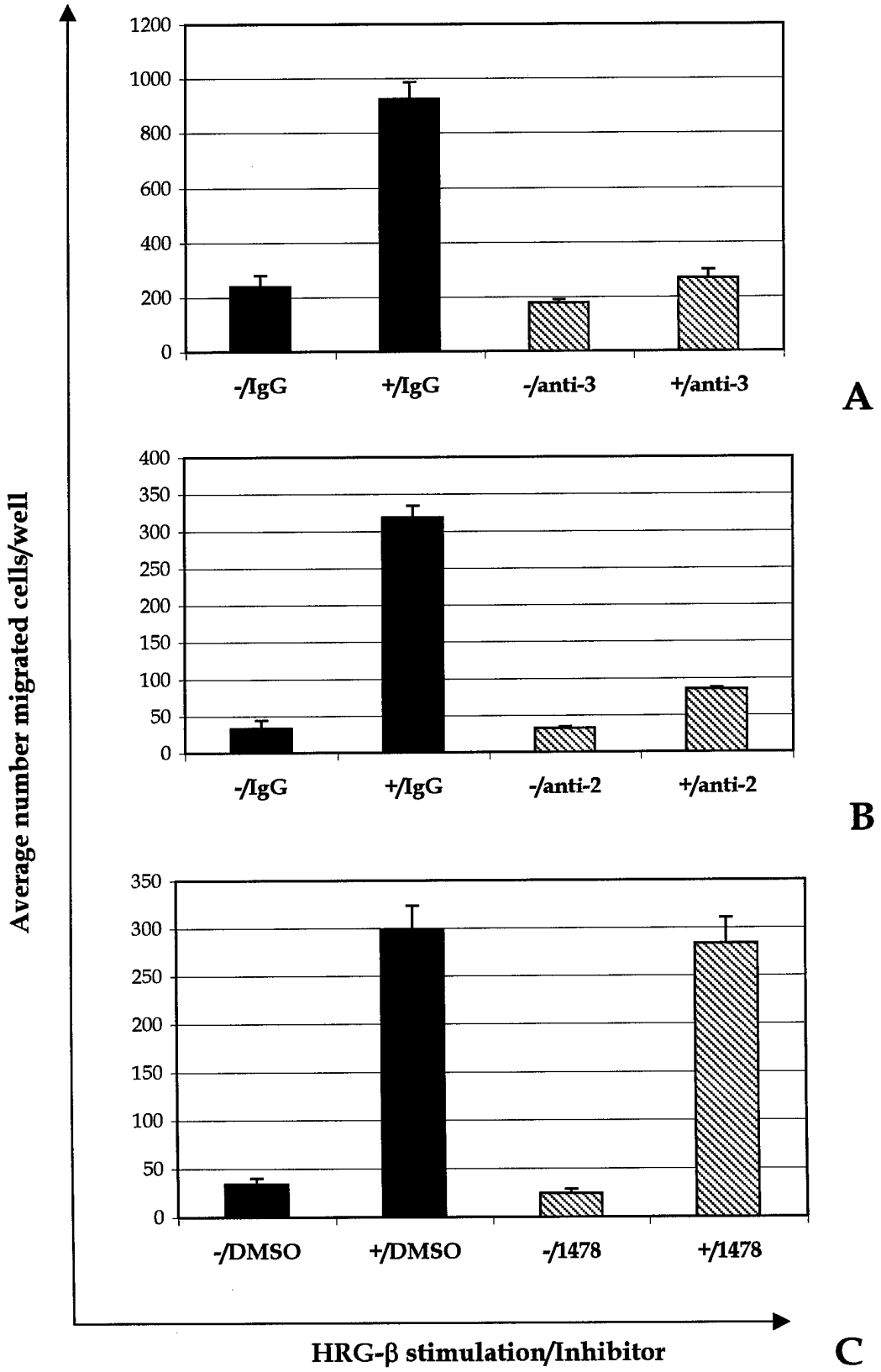
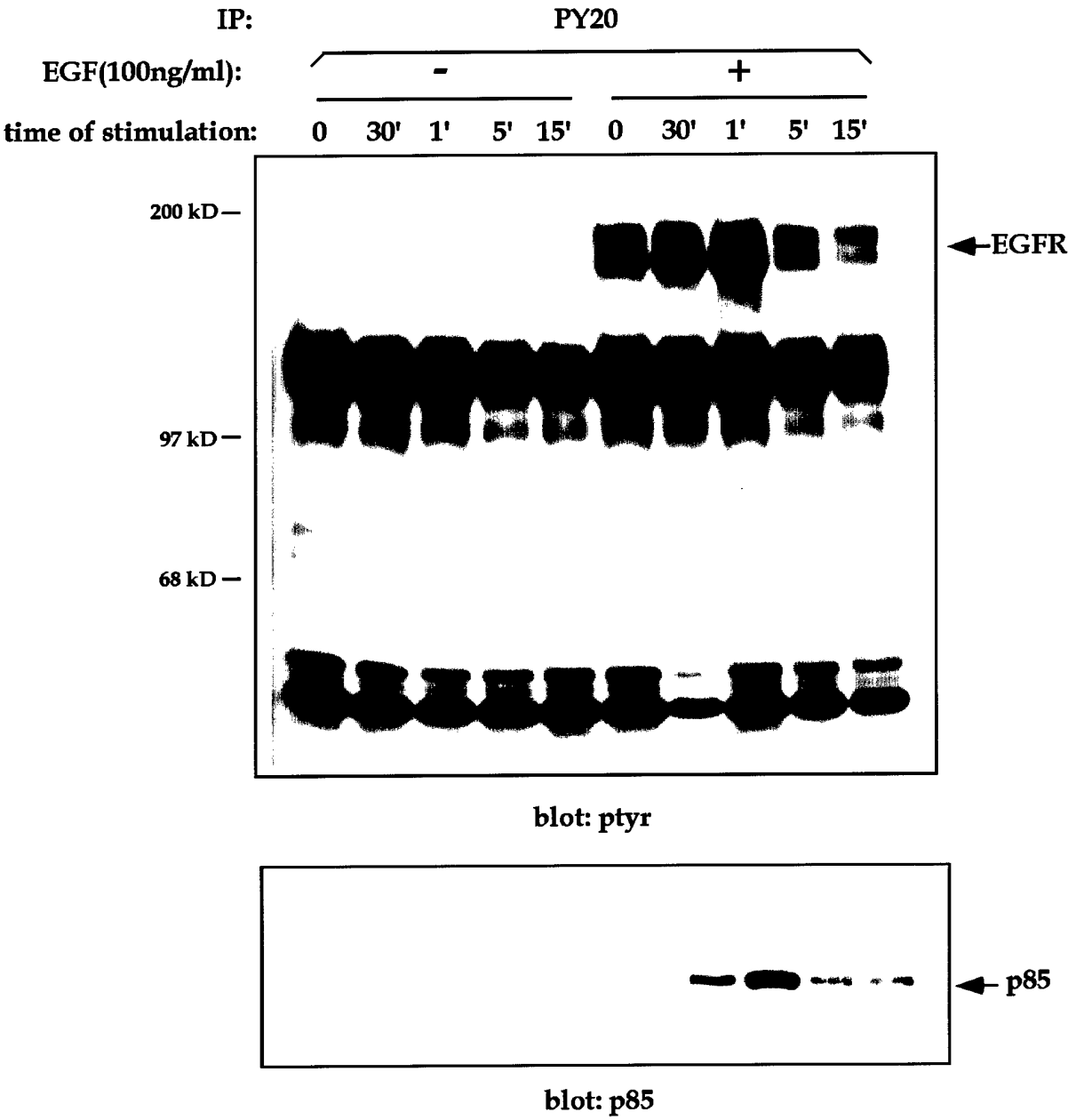
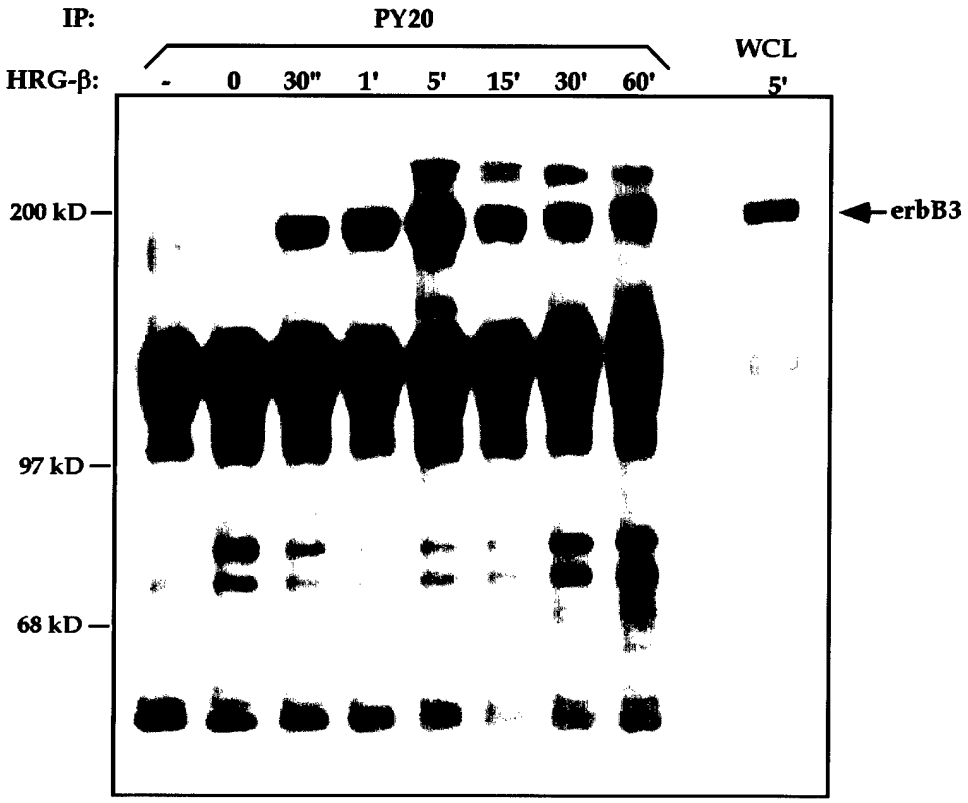

Average number migrated cells/well
1200
1000
800
600
400
200
0
-/IgG
+/IgG
-/anti-3
+/anti-3
A
400
350
300
250
200
150
100
50
0
-/IgG
+/IgG
-/anti-2
+/anti-2
B
350
300
250
200
150
100
50
0
-/DMSO
+/DMSO
-/1478
+/1478
C
HRG-β stimulation/Inhibitor

Figure 6


IP:
PY20
EGF(100ng/ml):
-
+
time of stimulation:
0 30' 1' 5' 15' 0 30' 1' 5' 15'
200 kD
97 kD
68 kD
EGFR
blot: ptyr
p85
blot: p85

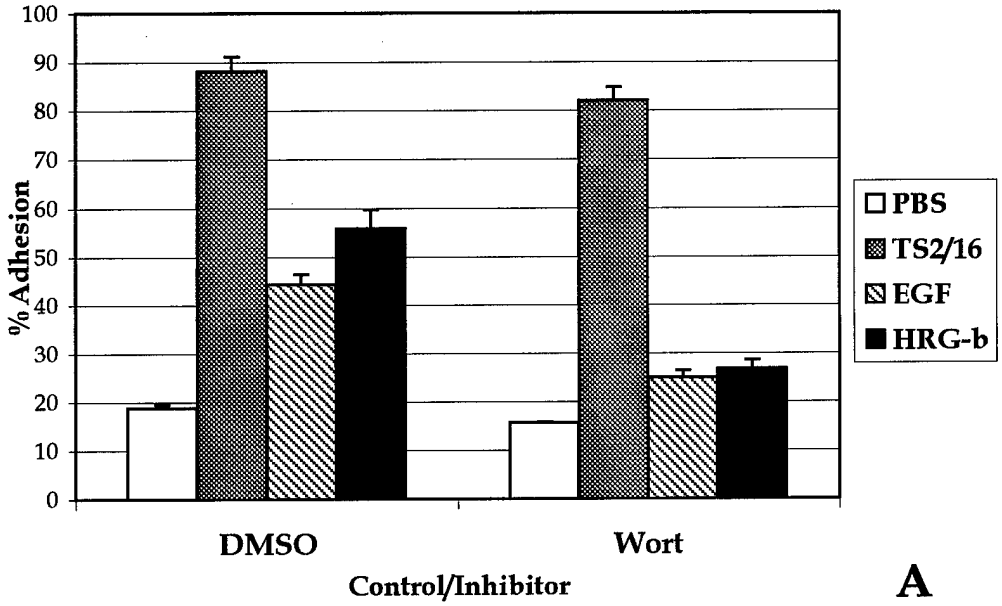
A

Figure 7

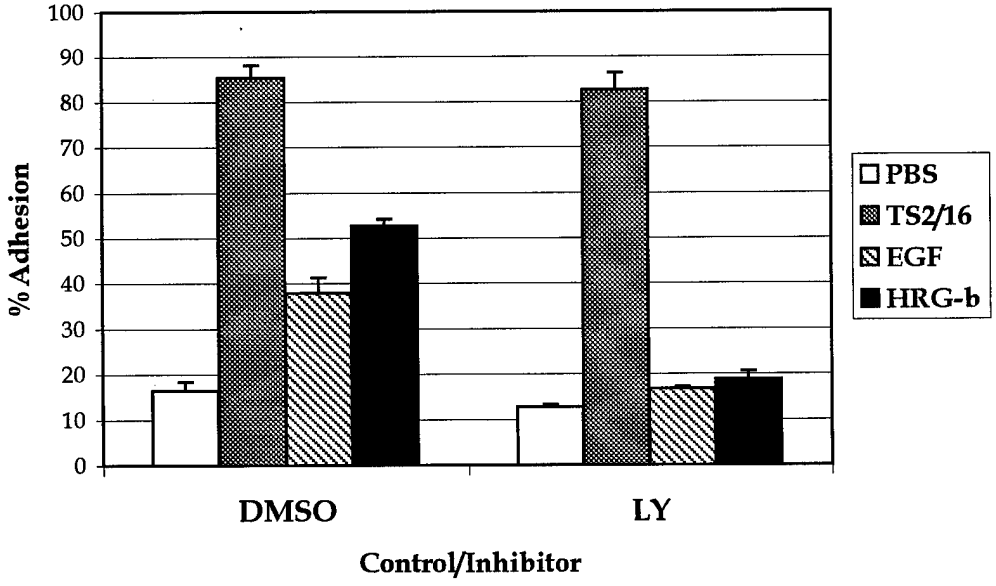


B

Figure 7 (cont)

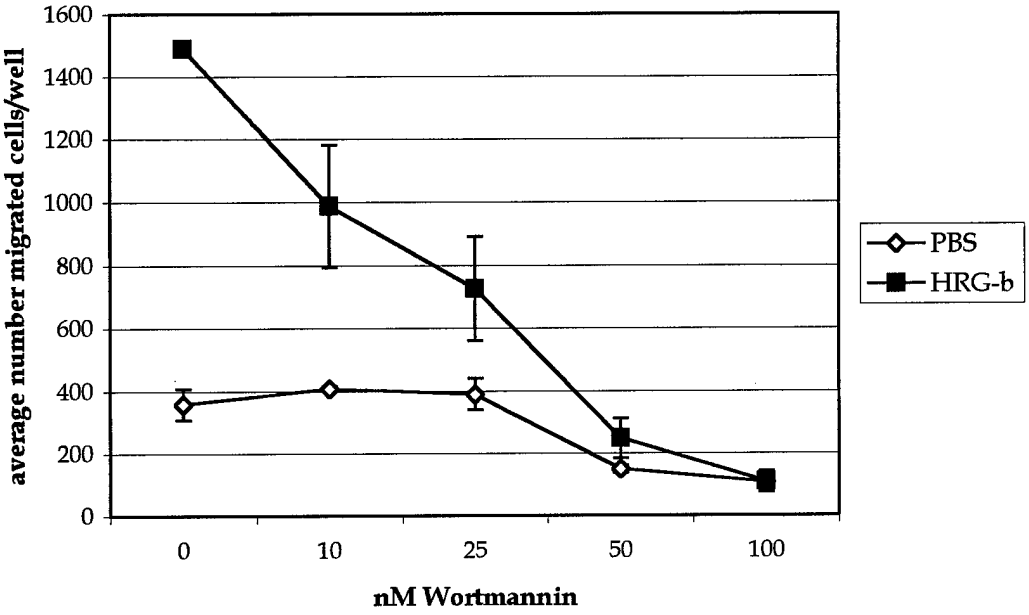

100
90
80
70
60
50
40
30
20
10
0
% Adhesion
PBS
TS2/16
EGF
HRG-b
DMSO
Wort
Control/Inhibitor

A

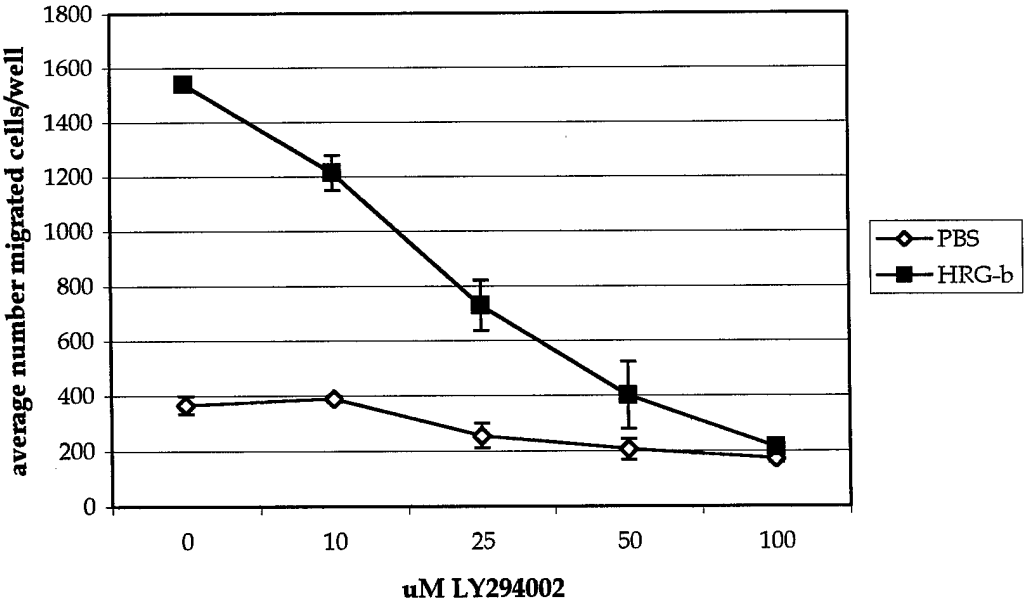

100
90
80
70
60
50
40
30
20
10
0
% Adhesion
PBS
TS2/16
EGF
HRG-b
DMSO
LY
Control/Inhibitor

B

Figure 8

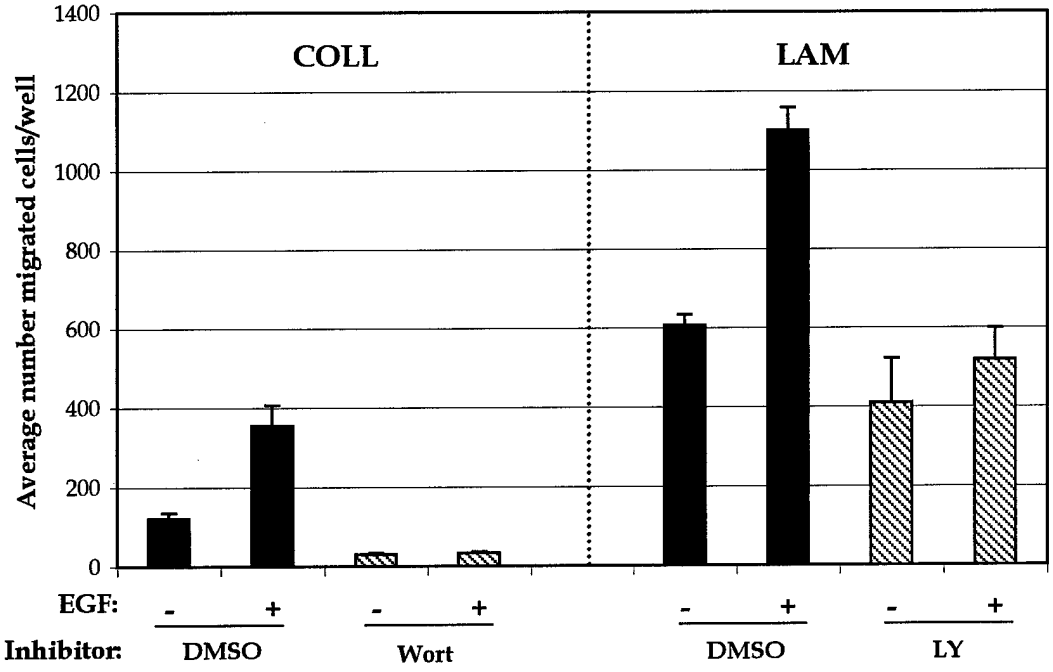


C



D

Figure 8 (cont)



E

Figure 8 (cont)

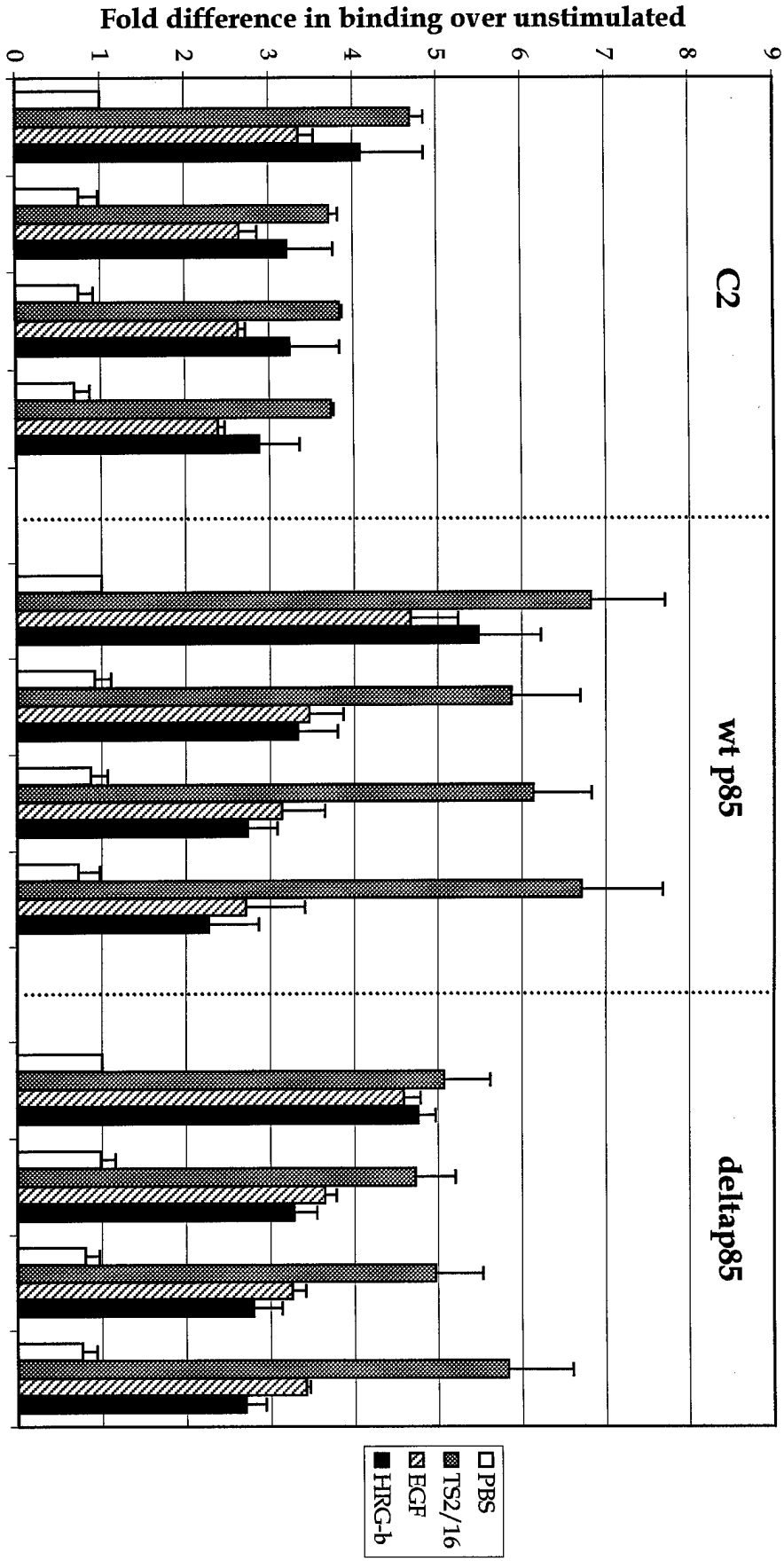
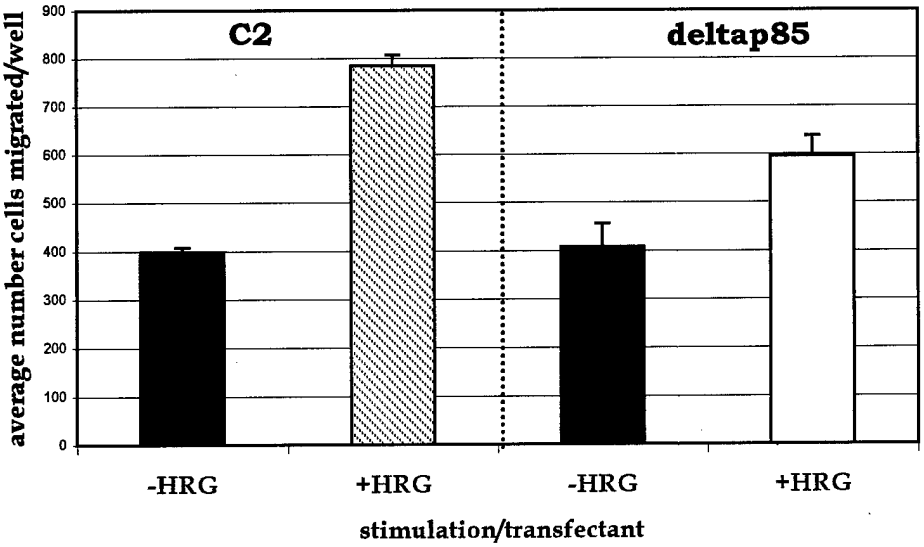
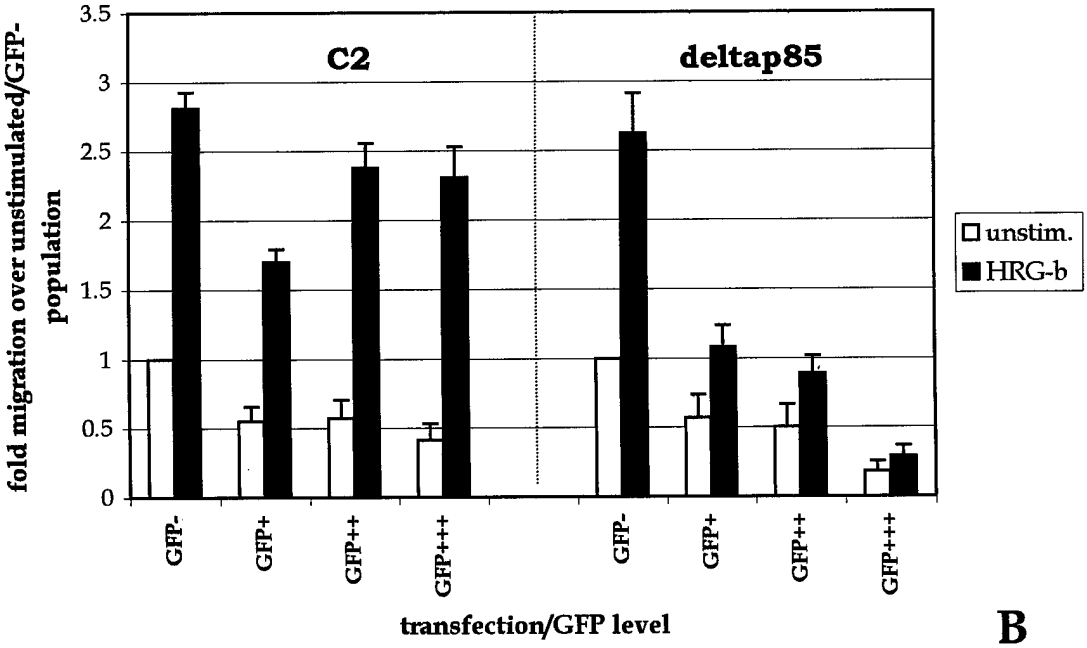

Fold difference in binding over unstimulated
0
1
2
3
4
5
6
7
8
9
C2
wt p85
deltap85
GFP-
GFP+
GFP++
GFP+++
Transfectant/GFP level
PBS
TS2/16
EGF
HRG-b

Figure 9


C2
deltap85
average number cells migrated/well
900
800
700
600
500
400
300
200
100
0
-HRG
+HRG
-HRG
+HRG
stimulation/transfectant

A


C2
deltap85
fold migration over unstimulated/GFP- population
3.5
3
2.5
2
1.5
1
0.5
0
unstim.
HRG-b
GFP-
GFP+
GFP++
GFP+++
GFP-
GFP+
GFP++
GFP+++
transfection/GFP level

B

Figure 10

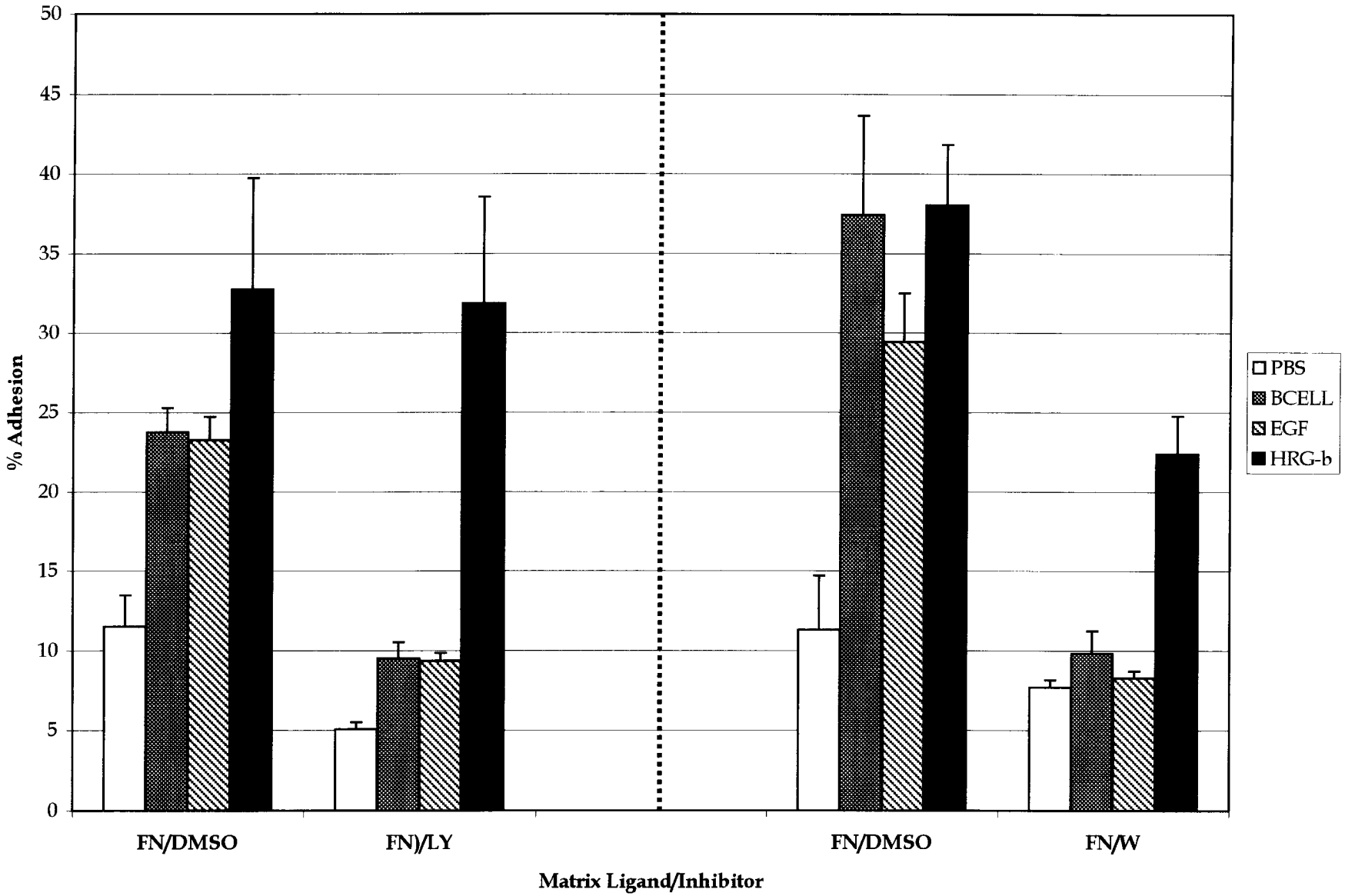
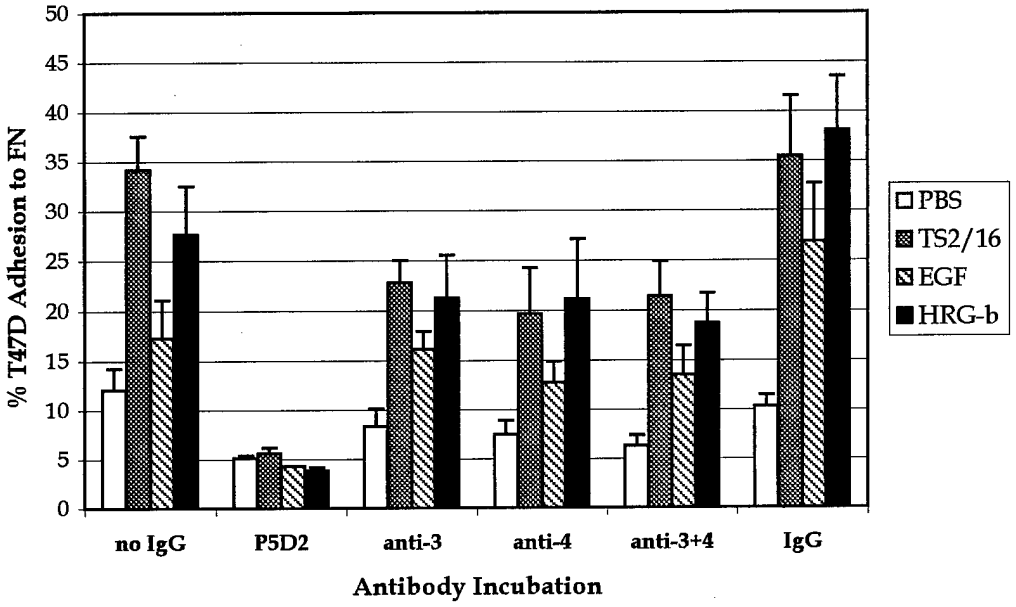
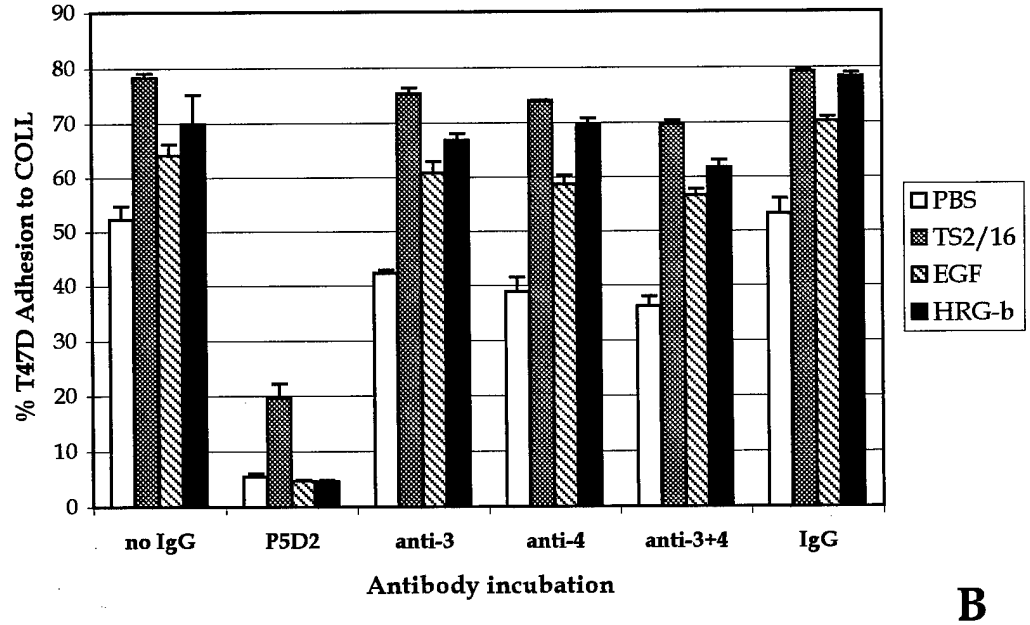

50
45
40
35
30
25
20
15
10
5
0
% Adhesion
PBS
BCELL
EGF
HRG-b
FN/DMSO
FN)/LY
FN/DMSO
FN/W
Matrix Ligand/Inhibitor

Figure 11

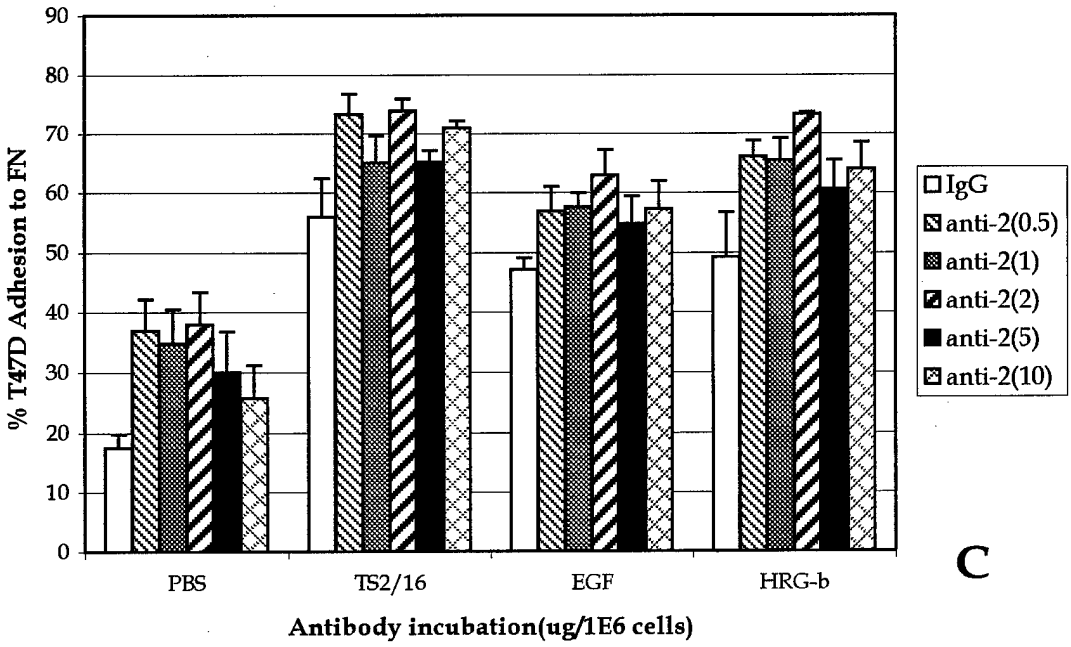

% T47D Adhesion to FN
50
45
40
35
30
25
20
15
10
5
0
no IgG
P5D2
anti-3
anti-4
anti-3+4
IgG
Antibody Incubation
PBS
TS2/16
EGF
HRG-b

A

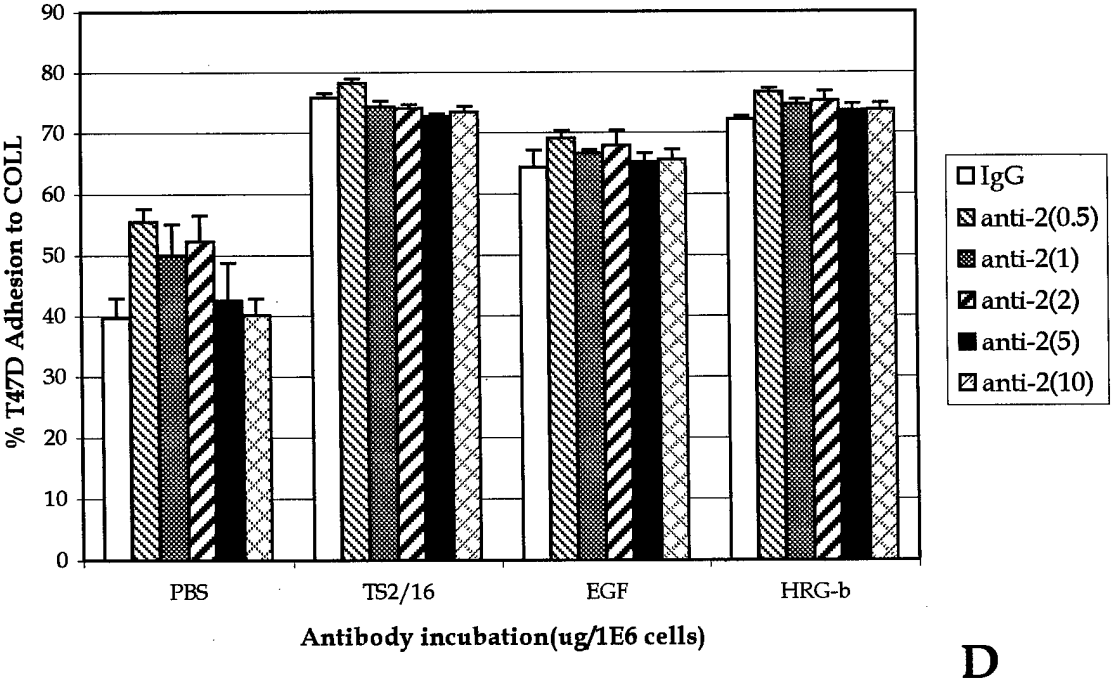

% T47D Adhesion to COLL
90
80
70
60
50
40
30
20
10
0
no IgG
P5D2
anti-3
anti-4
anti-3+4
IgG
Antibody incubation
PBS
TS2/16
EGF
HRG-b

B

Figure 12

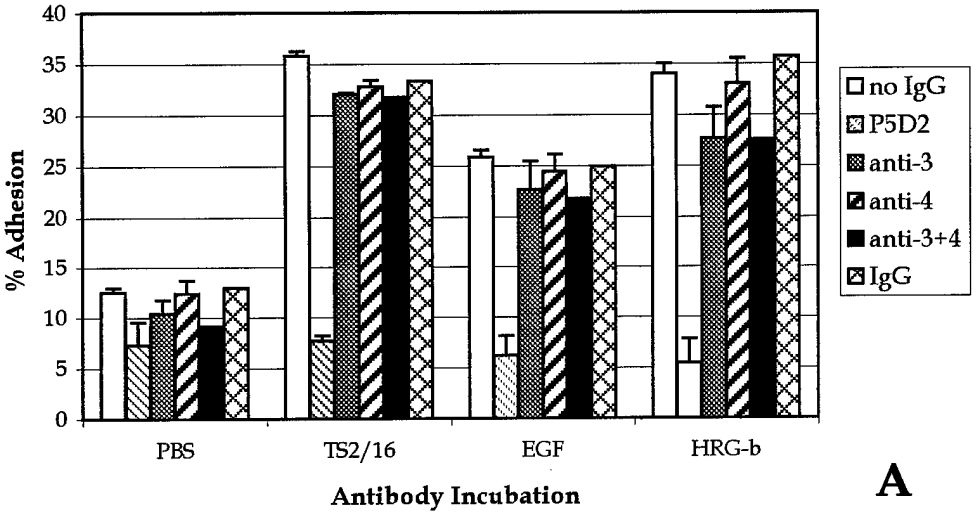


C

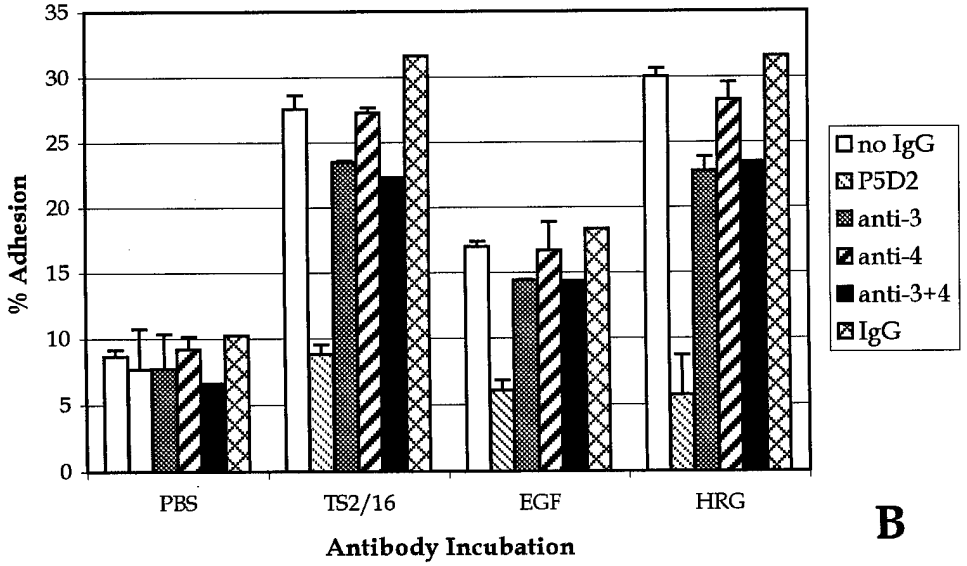


D

Figure 12 (cont)

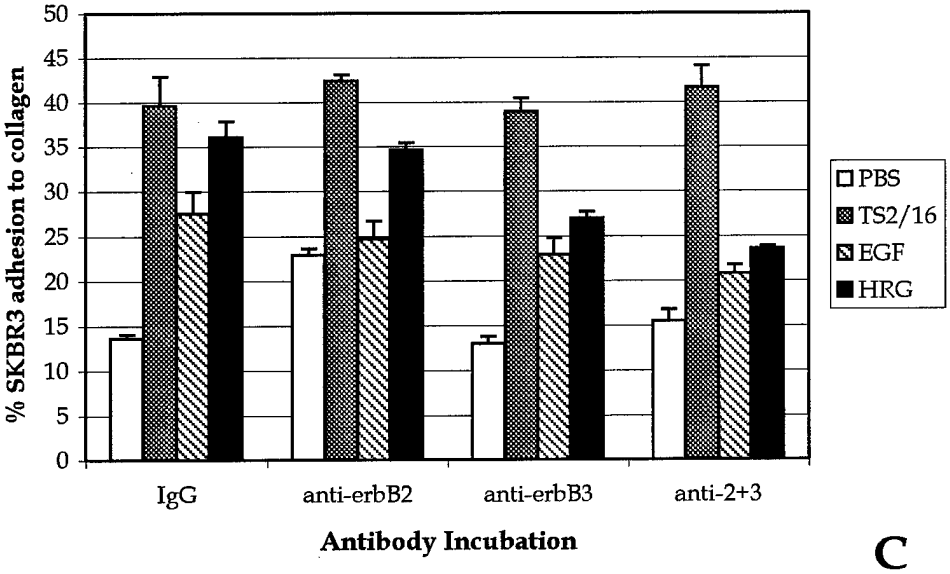

40
35
30
25
20
15
10
5
0
% Adhesion
PBS
TS2/16
EGF
HRG-b
Antibody Incubation
no IgG
P5D2
anti-3
anti-4
anti-3+4
IgG

A

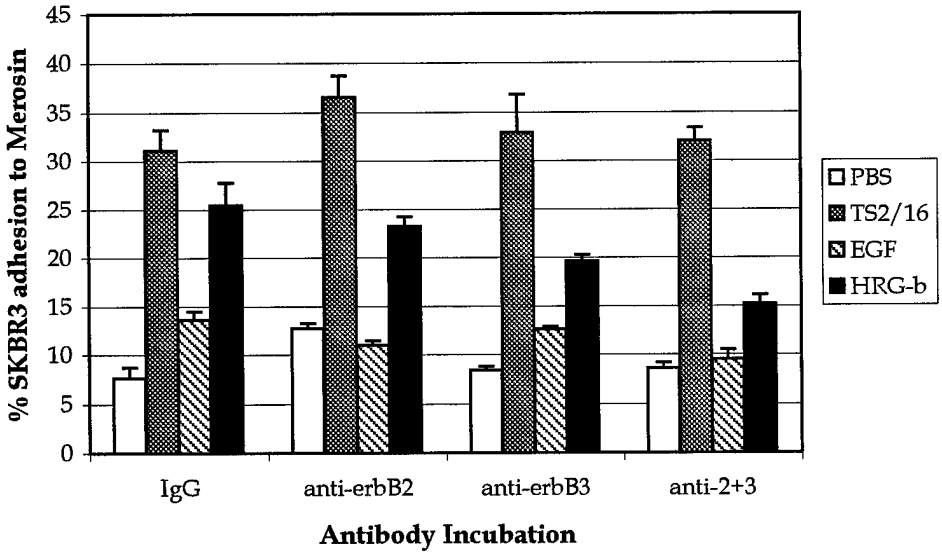

35
30
25
20
15
10
5
0
% Adhesion
PBS
TS2/16
EGF
HRG
Antibody Incubation
no IgG
P5D2
anti-3
anti-4
anti-3+4
IgG

B

Figure 13



C



D

Figure 13 (cont)